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CONTENTS

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The Inhibiting Effect of Ethyl Urethane on the Development of Lymphatic Leukemia in Rats*

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Hawkins and Murphy (2) reported in 1925 that animals anesthetized with ethyl urethane (ethylcarbamate) showed changes in the lymphoid system strikingly similar to those observed following a general exposure of the animal to x-ray.[1] The present investigation is a test of the effect of urethane on malignant lymphoid cells, as exemplified by a transplantable lymphatic leukemia and a lymphosarcoma of the rat. Previous investigations by Haddow and Sexton (1), in which they tested the effect of urethane on experimental animal tumors, showed a definite effect on the Walker Rat Carcinoma 256, but the action was more striking on leukemic cells. Paterson, Thomas, Haddow and Watkinson (9) reported that in human leukemia, urethane produces a fall in the white blood cell count, diminution in size of the spleen and involved lymph nodes, changes which "are remarkably similar to those obtained by standard methods of deep x-ray therapy."

MATERIAL AND METHODS

The transplantable lymphatic leukemia used in the tests has been carried in this laboratory since 1940 (5). The leukemic cells inoculated intraperitoneally cause the development of typical leukemia with marked increase in the circulating lymphocytes, and extreme involvement of the thymus and lymph nodes. Subcutaneous inoculation of the leukemic cells in the groin results in a rapidly-growing lymphosarcoma which may occasionally show metastases to the regional lymph nodes but rarely progresses into leukemia. At the time of the present tests about 84 per cent of the rats inoculated intraperitoneally developed leukemia which caused death in 8 to 10 days. Lymphosarcoma grew in approximately 86 per cent of the animals inoculated subcutaneously, with a fatal outcome between 12 and 18 days.

A 10 per cent concentration of ethyl urethane in 0.85 per cent salt solution was injected subcutaneously 5 times weekly and the doses varied from 25 to 100 mgm. per 100 gm. of body weight.

Effect of ethyl urethane on leukemia. In 8 tests, 79 rats inoculated intraperitoneally with leukemic cells were treated with subcutaneous injections of ethyl urethane. In the first experiment, treatment was started an hour after inoculation and the rats were given 100 mgm. of the drug per 100 gm. of body weight and this was repeated 5 times weekly. This gave complete protection against the development of leukemia but there was a question of how great a role the general toxic effect of the drug had on the result. In a second experiment the same dose of urethane was given 3 times weekly and this also resulted in complete protection. A third experiment in which the dose was reduced to 75 mgm. given 5 times weekly gave equally good protection. Five groups of inoculated rats were treated with 50 mgm. 5 times a week. There was slight, if any, manifest toxic effect from these doses of urethane, and the animals remained in good condition throughout the treatment period. As controls for these tests, 71 rats from the same strain were inoculated at the same time and with the same material but were given no treatment. The details and results of the individual experiments are given in Table I.

It will be noted that the degree of inhibition on the development of leukemia was almost as high with 50 mgm. of urethane as with the larger dose. In 6 of the experiments, treatment was initiated 1 hour after inoculation but the results in two experiments in which treatment was started 24 hours later were just as definite.

* A preliminary report on these observations was published in Science (8).

[1] It was noted in this study that rats, under urethane anesthesia, given an exposure to X-ray well under the lethal dose, invariably died between 7 to 14 days later.

Effect of urethane on lymphosarcoma.—In three experiments 60 rats were inoculated subcutaneously with leukemic cells. Of these, 30 rats were given injections of 50 to 75 mgm. of ethyl urethane per 100 gm. of body weight, starting 24 hours after inoculation, and the dose was repeated 5 times a week. In the treated rats 53.3 per cent failed to develop tumors; 16.6 per cent of the tumors that developed later retrogressed. Thus, only 30 per cent showed progressive tumors. This may be contrasted with the untreated controls where 86.6 per cent of the 30 rats had progressive tumors (Table II). The treated animals in which the tumors progressed showed a definite effect of the treatment in that they survived 12 to 14 days longer than the untreated controls (Table III).

TABLE III: EFFECT OF ETHYL URETHANE ON LYMPHOSARCOMA

Urethane dosage	Total number of treatments	Number of rats	No growth	Retrogressive	Progressive	Average number of days lived
			%	%	%	
50 mgm. 4-5 times weekly	16	10	50	10	40	29*
25 mgm. 4-5 times weekly	16	10	0	70	30	27
Controls	—	10	1	0	90	15

* Killed with large tumors.

TABLE I: RESULTS OF INDIVIDUAL EXPERIMENTS

Experiment number	Number of rats	Amount urethane per 100 gm. body weight	Time after inoculation	Frequency per week	Negative	Controls	
						Number of rats	Negative
		mgm.	*hours*		%		%
1	10	100	1	5	100	10	30
2	10	100	1	3	100	10	30
3	10	75	1	5	100	10	0
4	10	50	1	5	100	*	
5	10	50	1	5	100	10	20
6	9	50	1	5	88.8	10	10
7	10	50	24	5	80	11	18.2
8	10	50	24	5	100	10	10
Total 8	79				91.1	71	16.9

* Controls for this group were the same as those in Experiment 2.

Effect of ethyl urethane on white blood cells, lymphoid organs and adrenals.—Blood counts on groups of animals from the foregoing experiments confirm our earlier observations (2). The urethane-treated rats showed a rapid fall in the circulating lymphocytes, reducing the number of both normal lymphocytes and leukemic cells to approximately a third of the previous level.

Table IV shows the average weights of the lymphoid organs and adrenals of 80 rats treated with urethane and the same data from 71 untreated controls. It will be noted that, in the first group of treated animals developing leukemia, the shrinkage of the thymus and spleen was less pronounced than in the other groups and there was no significant hypertrophy of the adrenals. In as yet an unpublished study of the effect of the growth of rat lymphosarcoma on the lymphoid organs, a pronounced regression of the thymus was noted. The controls of group 3 give an example of this reaction with 3 of the animals showing complete atrophy of the thymus. Averaging the weights of the several organs from 80 urethane-treated rats and the 71 controls gives the following results. The thymus was present in only 58.7 per cent of the treated rats; when present, the average weight of this organ was a third of that of the average weight of the thymus in the controls. The cervical nodes and spleens in the treated animals were reduced approximately by a half.

It will also be noted in Table IV that the adrenals of the controls of group 4 animals, resistant to the lymphosarcoma, are larger than the average for the other untreated rats. This same condition has been observed in a large number of rats in another study, the details of which will be published later. With the exception of group 1, the urethane-treated animals were found to have definitely enlarged adrenals, approximately 40 per cent larger than these organs from the untreated controls.

TABLE II: EFFECT OF ETHYL URETHANE ON LYMPHOSARCOMA

	Number of rats	No growth	Retrogressive tumors	Progressive tumors
		%	%	%
Treated with urethane	30	53.3	16.6	30.0
Controls	30	3.3	10.0	86.6

Rats treated with 50-75 mgm. ethyl urethane per 100 gm. body weight, 5 times weekly. First treatment 24 hours after inoculation.

DISCUSSION

It has previously been shown in this laboratory that adrenalectomized rats have a definitely in-

creased susceptibility to transplanted lymphatic leukemia (6, 7) and that adrenal cortical and pituitary adrenotropic hormones have a definite inhibiting effect on the development of this disease (7). In an as yet unpublished study it was noted that rats with growing lymphosarcoma have hypertrophy of the adrenals (25 per cent) and that rats which develop resistance to this growth show even more pronounced enlargement of these glands (34.4 per cent). In the present investigation, definite enlargement of the adrenals (40 per cent) is noted in the rats treated with ethyl urethane. Attempts have been made to determine if the effect of this treatment on leukemia was due to the stimulation of the adrenals rather than to direct action on the leukemic cells. Unfortunately, adrenalectomized rats do not withstand even a small amount of urethane, so the answer to the question must await a different approach.

It was demonstrated in this laboratory that alkalosis induced by injections of sodium bicarbonate has a destructive effect on the lymphoid system quite comparable to that resulting from general x-ray exposure (3, 4). As noted above, ethyl urethane causes a similar destruction of the lymphoid system, and this chemical also induced pronounced uncompensated alkalosis (2). These observations suggest the possibility, perhaps remote, that the urethane effect may depend on the induced alkalosis. This question is now under investigation.

SUMMARY

Among 79 rats inoculated with leukemic cells and treated with ethyl urethane in doses ranging from 50 to 100 mgm. per 100 gm. of body weight, repeated 3 to 5 times weekly, 91.1 per cent failed to develop the disease. In 71 inoculated but untreated controls only 16.9 per cent were resistant. A similar inhibiting effect was found in the treatment of rats with lymphosarcoma. Among 30 rats given ethyl urethane only 30 per cent developed progressive tumors while 86 per cent of the 30 controls died with large tumors. The thymus, lymph nodes and spleen in rats receiving urethane treatment were reduced to a fraction of their normal weights. The adrenals of these animals increased 40 per cent in weight over that of the normal glands.

TABLE IV: WEIGHTS OF LYMPHOID ORGANS AND ADRENALS IN RATS WITH LEUKEMIA

	Groups	Number of rats	Thymus	Cervical nodes	Spleen	Adrenals
			mgm.	*mgm.*	*mgm.*	*mgm.*
1	Treated with urethane	3	239.8	172.4	536.9	32.6
	Controls	33	459.8	170.5	817.3	30.9
			RESISTANT TO LEUKEMIA			
2	Treated with urethane	47	105.7 (23 no thymus)	90.0	624.5	43.1
	Controls	8	344.3	217.9	1408.4	29.0
			WITH PROGRESSIVE LYMPHOSARCOMA			
3	Treated with urethane	5	90.6 (3 no thymus)	76.2	374.6	43.9
	Controls	17	101.8 (3 no thymus)	121.5	1333.3	29.4
			RESISTANT TO LYMPHOSARCOMA			
4	Treated with urethane	15	65.0 (7 no thymus)	50.0	411.5	41.8
	Controls	3	147.0	177.8	960.9	34.8
			NORMAL RATS			
5	Treated with urethane	10	65.2	94.8	475.9	38.6
	Controls	10	213.8	255.6	1004.1	29.3
				Average weights		
	Total treated with urethane	80	97.9 (33 no thymus)	87.9	547.0	42.0
	Total controls	71	322.5 (3 no thymus)	176.4	1052.5	30.3

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Electron Microscope Study of Chicken Tumor Cells*

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Chicken tumors of spontaneous origin are characterized by the fact that most of them can be transmitted to new hosts in the absence of cells (11). From the beginning it was apparent that the agents transmitting the tumors were of relatively small size since they were not retained by Berkefeld filters of medium porosity (30, 31, 32, 33). On the basis of filtration experiments using collodion membranes of graded pore sizes it was estimated that the probable diameter of the agent of chicken tumor I was approximately 70 mμ (15). A close agreement with this value was obtained from determination of the speed of sedimentation of the tumor-producing factor in high centrifugal fields (6, 16, 20). Concentration and purification of the agent by means of differential centrifugation gave preparations of high potency (6, 7). Subsequently, it was found that these active fractions were contaminated with a normal, sub-microscopic, constituent of cytoplasm of nearly the same size as the tumor agent (8).

Because of their small size, the agents causing chicken tumors cannot be resolved by means of light microscopy; purified fractions containing them have appeared particulate under dark-field illumination (6) but the presence in large amounts of a normal contaminant made these observations inconclusive. The light microscope has also failed to disclose any inclusion bodies or other abnormalities in the tumor cells that might be considered as indicative of the presence of the filterable agent.

In the present investigation advantage has been taken of the fact, demonstrated in an earlier study (28), that cells of most types in tissue culture spread out to a thinness which makes electron microscopy possible. Chicken sarcoma cells are among the elements which behave in this way. A detailed study has now been made of those from two tumors, namely Chicken Tumor I and Chicken Tumor 10. The electron microscope has revealed in the cytoplasm of these cells, small bodies of a size predicted from centrifugation and filtration tests with the tumor agents. The disposition of these bodies within the cell appears to be distinctive for the kind of tumor examined. No similar structures have been found in an extensive study of homologous normal cells obtained from tissues of adult fowls or from chick embryos, or from the buffy coat of chicken blood. The evidence is strong that the small bodies in the neoplastic cells are the individual tumor-producing entities.

MATERIALS AND METHODS

Chicken Tumor I.—Since 1909 this tumor has been maintained in the Rockefeller Institute either by implanting the sarcomatous tissue in Plymouth Rock hens or pullets, or by the injection of tumor filtrates or desiccates. The histology and general characteristics of the tumor have been the subject of repeated studies (21, 29, 30). It is a myxosarcoma of rapid growth, which generally kills the host in 10 to 20 days, and metastasizes most frequently to the lungs, heart, and liver. Growing tumors actively invade and replace the surrounding tissues and, as a rule, produce large quantities of hyaluronic acid (19). Histologically, two types of cells can usually be recognized: (a) spindle-shaped, fibroblast-like cells which lie singly or in bundles, and (b) large, polymorphous cells of the macrophage type. Giant multinucleated cells of both types are encountered not infrequently. Cell-free extracts of the tumors produce growths of the same sort.

Chicken Tumor 10.—This tumor arose in a stock chicken in this laboratory and was transplanted by Murphy and Sturm.[1] During the first five years of propagation it grew very slowly, often requiring 8 months to a year to kill the animal. It was transmitted with difficulty by desiccates and only one doubtful result from many attempts was obtained with filtrates. At times the growth was more rapid

* The observations recorded in this paper were first presented at the 37th Annual Meeting of the American Association for Cancer Research, Inc., Atlantic City, New Jersey, March 12, 1946, and abstracted in *Cancer Research* (12).

[1] No detailed report has been made on Chicken Tumor 10. It was first referred to in 1931 by Murphy and Sturm (25) and briefly described by these authors when it was utilized as a source of the inhibitor factor (27). An illustrated description of this tumor has appeared in a paper by Burk and his co-workers (3).

but even at these periods metastasis was rare (27). It was demonstrated in this laboratory that extracts of this tumor inhibit the agent of Chicken Tumor I (25), and Andrews (1) reported that the serum from chickens bearing this tumor neutralizes the agents of Chicken Tumor I and also that of a tumor known as Mill Hill 2. Murphy and his coworkers demonstrated that inactive extracts of Chicken Tumor I would regularly produce tumors if an inhibiting factor was removed from the preparation (25, 26). The possibility that the difficulty in transmission of Chicken Tumor 10 was due to the presence of an excessive amount of an inhibitor was investigated by Claude (10), who found that an active agent could be separated from the associated inhibitor by high-speed centrifugation. In recent years such separation has become unnecessary, the tumor constantly yielding filtrates on ordinary extraction which have a tumor-producing power in dilutions up to 10^6. By early transmission to young pullets the growth rate of this tumor has progressively increased till now it equals that of Chicken Tumor I. Metastases appear in the gizzard, the heart, and less frequently in the spleen and liver. The tumor grows into nodular masses of firm, characteristically white tissue. Histologically, it is a sarcoma composed of spindle-shaped rather plump cells, closely packed in bundles. Mitoses are numerous. The growths are often typically fissured with sinus-like cavities, lined with a thin endothelium.

Material for tissue culture.—The explants for tissue culture were taken from rapidly growing tumors. In order to exclude so far as possible the presence of normal cells, and to avoid possible interference from antibodies and inhibitors known to occur in older hosts (14), the chicken tumors providing the explants were grown on the chorioallantoic membrane of developing chick embryos (24). Such tumors were started either with 0.1 cc. of a suspension of fresh tumor pulp in Tyrode's solution, or with 0.2 cc. of a concentrated Berkefeld filtrate placed on the membrane of 10 day old embryos. After 5 to 7 days the tumors had developed, in some cases to 2.0 cm. or more in diameter and were removed. As a rule, the best tissue culture preparations were obtained from this source.

Control, normal cells were fibroblasts and macrophage-like cells obtained from the skeletal muscles of adult hens, or of chick embryos. Blood macrophages for tissue culture were obtained by centrifuging the blood of normal chickens, and explanting small fragments of the buffy coat.

Technique of tissue culture.—Tumor and control normal cells were grown *in vitro* essentially according to the technic described in detail in a previous paper (28). For growth of short duration the cells were cultured according to the classical hanging-drop method, either in serum-saline mixtures or under nutrient plasma clot. For sustained growth the cells were cultured under plasma clot and circulating nutrient fluid, on cover glasses placed in roller flasks. The clot was made of coagulated chicken plasma; the nutrient fluids were mixtures in varying proportions of horse or chicken serum, chick embryo extract, and Tyrode's solution.

The blood of apparently normal fowl is often found to possess in varying degrees the power to suppress or inhibit the free tumor agents (14), and it is possible that such serum antibodies might interfere with the proliferation of the chicken tumor agent in tissue cultures and even restrain the growth of chicken tumor cells. With this consideration in mind, the blood of normal chickens was tested beforehand by injecting intracutaneously in adult hens serial dilutions of mixtures of serum and active tumor filtrates. Chickens whose blood showed little or no neutralizing power were selected as donors for tissue culture media.

Preparation of cells for electron microscopy.—The method by which the extended cells can be transferred from the culture to the supporting wire mesh of the electron microscope has been described in a preceding paper (28). Briefly, it consists in coating beforehand the glass surface designed to support the culture with a thin film of plastic (Formvar). The cells, which can extend over the plastic as well as on the uncoated glass surface, are thereafter fixed, lifted from the glass along with the film of plastic to which they firmly adhere, and transferred, under water, to the conventional disc of wire mesh.

Cells for electron microscopy were fixed by exposing the culture to osmium tetroxide for periods ranging from a few minutes to 24 hours, either by osmic vapors, or by covering the culture with a 2 per cent solution of osmic acid in 0.85 per cent NaCl solution or water. The preparations were rinsed in distilled water for varying lengths of time before mounting on the wire mesh and drying.

All the observations and micrographs presented in this paper were made with an R.C.A., type E.M.U., electron microscope.

OBSERVATIONS

The appearance and behavior of chicken tumor cells in tissue culture have been described repeatedly. Two different cell types have been generally recognized, namely, fibroblasts and macrophages, together with giant cells and degenerative forms derived from both cell types. It

is still an open question whether the malignant condition is restricted to one cell type only or whether both macrophages and fibroblasts can harbor the agent which is responsible for the disease, and serve as medium for its proliferation and transmission. Divergent views concerning this point have been held by various workers (2, 4, 13, 17, 18, 22, 34) but, thus far, it does not seem that conclusive evidence has been presented to permit the exclusion of one or the other cell type from taking part in the malignant process. The controversy would lose its significance if it were demonstrated, as it has been suggested (5, 23), that fibroblasts and macrophages are but two forms of the same cellular element.

Histologically, chicken tumors have been found to possess the properties characteristic of malignant tumors generally *i. e.*, a typical and unrestricted growth, invasiveness for surrounding normal tissues etc. Cytologically, likewise, chicken tumor cells have been shown to exhibit the cellular features commonly found in tumor cells, such as atypical and multipolar mitosis, uneven division, and increased basophilia of the cytoplasm.

Results of Electron Microscopy

Normal cells.—In the present work the number of control cultures and of normal chicken cells examined was considerably greater than that of chicken tumor cells. Almost invariably, cultivation of tumor cells was accompanied by a parallel series of normal cells grown and treated under similar conditions. To this may be added, as source of additional evidence, the numerous normal chicken and chick embryo cells grown and examined by one of us (K.R.P.) under various conditions during and following this investigation.

Normal chicken fibroblasts, as they appear under the high magnification of the electron microscope, have been illustrated in a preceeding paper (28). For this reason, and also because the present study of chicken tumors deals largely with cells presenting the general morphology of macrophages, illustrations in this report will be concerned mostly with cells of the macrophage type.

The cell, a sector of which is shown in Fig. 1, was part of a culture grown from a fragment of the clotted buffy coat of centrifuged, presumably normal, chicken blood explanted to a Formvar-coated cover glass, under undiluted chicken serum. Under the light microscope the circularly extended cell has the appearance of a typical macrophage. Its nucleus, situated next to the lower left-hand corner of Fig. 1, is not shown and was not visible in the electron microscope since it rested on one of the wires of the supporting screen. The regularly spherical, opaque bodies which appear in a group in the same lower left corner of the micrograph are fat bodies or Golgi bodies. The slender elongated structures which radiate from the center of the cell are mitochondria. The open region between the center of the cell and the cell margin is occupied in part by a material of finer texture and lesser density, seemingly made up of small bodies or vesicles of rather uniform size. The latter cell constituent may correspond at least in part to particulate components of the ground substance (microsomes).

In addition to those constituents of normal cells illustrated in Fig. 1, chicken tumor cells have been found to contain in variable abundance small bodies, characteristic in size and appearance, which are considered to represent the causative agents of the disease. Such bodies have not been detected in normal chicken cells thus far examined by electron microscopy.

Chicken Tumor 10.—The cell, a portion of which is illustrated in Fig. 2, was part of a culture derived from a Chicken Tumor 10 growing in a Plymouth Rock pullet. Hanging-drop cultures were prepared in the usual manner by placing tumor explants on coated cover glasses and incubating under chicken plasma clot and chick embryo-horse serum nutrient. Cultures showing active migration and suitably extended cells were fixed 4 days later and mounted for examination in the electron microscope.

The micrograph shows the inclusions commonly found in normal cells, *i.e.*, scattered Golgi or fat bodies, mitochondria, and constituents of the ground substance. In addition and of special interest is the presence of patches of greater density which vary in size and shape and which thus far have been detected only in cells of Chicken Tumor 10. It is readily seen in Fig. 2 that these patches result from the aggregation of small elements of uniform size and shape and which appear not unlike growing colonies of staphylococci. In the case of Chicken Tumor 10, it might be assumed that these formations have resulted from the growth and subdivision of their constituting elements. From measurements on enlarged micrographs the diameters of the individual bodies are found to vary from 60 to 70 mμ. The presence of small bodies of this size in tumor cells but not in normal cells, their arrangement in "colonies," a feature apparently typical for Chicken Tumor 10, support the conclusion that the small elements observed represent the causative agent of the tumor.

Chicken Tumor I.—Fig. 3 represents the tip and margin of a cell from Chicken Tumor I. The culture providing the cell was grown for 48 hours under chicken plasma clot and diluted chick em-

bryo extract. The preparation was then fixed and mounted for examination in the electron microscope. The outstanding feature of the micrograph illustrated in Fig. 3 is the presence of numerous and conspicuous small bodies disposed along the cell margin, which appear singly, frequently in pairs, or in small rows of 4 or 6. Considerations to be discussed later suggest that these small elements represent the causative agent of Chicken Tumor I.

The disposition of the agent in pairs or in small loose groups seems typical for Chicken Tumor I. It is further illustrated in the next micrograph (Fig. 4). In this particular case, the body of the tumor cells in the tissue culture was removed, possibly during the preparation of the mount, leaving on the film adherent patches of the cell membrane together with series of bodies similar to those seen in the intact cell illustrated in Fig. 3. Measurements on enlarged micrographs indicate

FIG. 1.—Normal cell (control). Sector of extended macrophage showing common cell constituents previously described, *i. e.* Golgi bodies, mitochondria, and components of the ground substance. *Cultures*: Explant from the buffy coat of centrifuged blood from a young Plymouth Rock chicken, prepared as "lying drop" under undiluted chicken serum, and incubated for 3 days at 38° C. *Fixation*: Culture washed with Tyrode's solution, fixed by covering with 2 per cent osmic acid solution for 24 hours, and rinsed for 20 minutes in distilled water. Electron micrograph taken at 1,960 ×, enlarged to 5,900 ×.

FIG. 2.—Chicken Tumor 10. Portion of a cell showing in addition to normal constituents, patches or "colonies" of the causative agent of the tumor. *Culture*: Explant of a 13 day old tumor (Chicken Tumor 10) grown in a Plymouth Rock pullet; prepared as "hanging drop" under clot of chicken plasma and nutrient mixture composed of horse serum, chick embryo extract, and Tyrode's solution. *Fixation*: culture used after four days incubation at 38° C.; fixed by covering with 2 per cent osmic acid solution in water for 3 hours, and rinsed for 15 minutes in distilled water. The picture shows clearly the individual bodies, 60 to 70 mμ in diameter, which make up what, at lower magnifications, appear as patches of greater density. Electron micrograph at 1,960 ×, enlarged to 10,700 ×.

FIG. 3.—Chicken Tumor I. Tip and margin of a cell showing the causative agent of the tumor as it occurs singly, in pairs, or in rows of 4 or 6. These bodies are approximately 70 to 85 mμ in diameter. *Culture*: Explant of a 5 day old tumor growing on the chorioallantoic membrane of a chick embryo. Culture prepared as "hanging drop" under clot of chicken plasma and nutrient consisting of chick embryo extract, diluted with Tyrode's solution. *Fixation*: Culture used after incubation for 48 hours at 38° C. Fixed by covering with a 2 per cent solution of osmic acid, for 20 hours, Electron micrograph taken at 1,960 ×, enlarged to 15,800 ×.

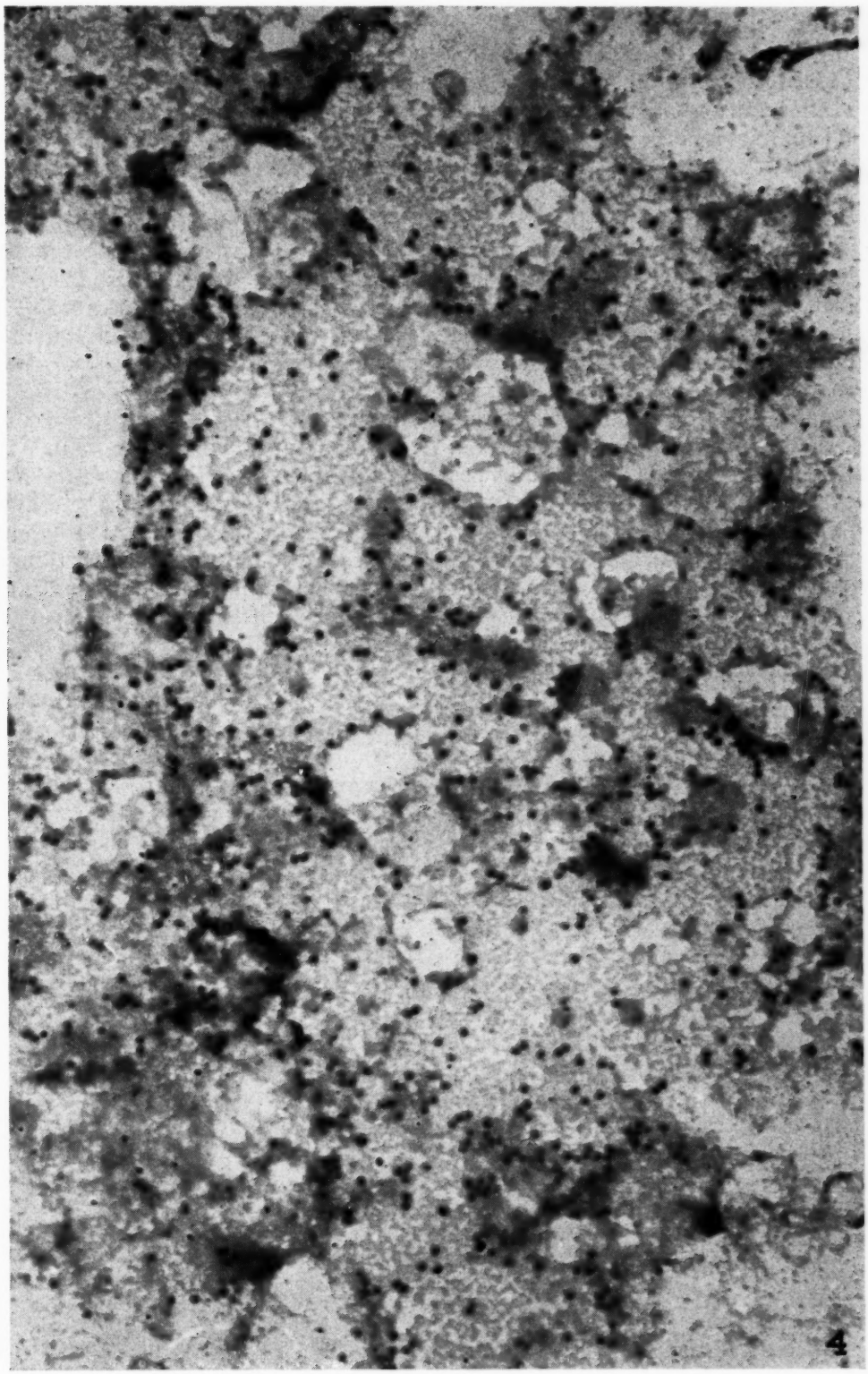

FIG. 4.—Chicken Tumor I. The picture shows the Chicken Tumor I agent appearing singly, in pairs, or in short strings; note the frequent occurrence of paired elements. The size of these bodies ranges from 70 to 85 mμ. The background shows portions of the cell which remained adherent to the plastic film. *Culture*: Explant of a 14 day old tumor grown in a young Plymouth Rock pullet. Culture prepared as "lying drop" by placing the tissue fragment on a cover glass and covering with a drop of undiluted chicken serum. *Fixation*: Preparation rinsed with Tyrode's solution, fixed for 30 minutes by covering with a 2 per cent solution of osmic acid in water, and washed for 10 minutes in distilled water. Electron micrograph taken at 2,280 ×, enlarged to 11,500 ×.

that the size of these bodies, in different preparations, ranges from 67 to 84 mμ in diameter. Close examination of the preparation shown in Fig. 4 reveals that paired elements are numerous and probably more frequent than could be expected if the occurrence in pairs were determined by chance alone. In the present case it seems reasonable to assume that these bodies are capable of dividing and that their appearance in pairs and in short strings is the result of a single, or a few successive subdivisions. It is conceivable that the arrangement found in Chicken Tumor I cells would be achieved if the process of successive duplication of the chicken tumor agent were oriented in space according to one plane, whereas the disposition obtained in Chicken Tumor 10 could result from subdivision of the agent at random, or according to planes oriented, alternatively, at right angles to each other.

DISCUSSION

By means of electron microscopy it has been possible to detect in the cells of two chicken tumors, but not in homologous cells, the presence of small bodies of relatively uniform size. Those observed in Chicken Tumor I range in size approximately from 67 to 80 mμ, which is in fair agreement with values previously obtained from filtration and centrifugation experiments (15, 16) with the agents causing this tumor. Those found in the case of Chicken Tumor 10 appear to be practically identical in size with those of Chicken Tumor I, though possibly somewhat smaller.[2] In both cases the bodies are in the form of regular spheres, but no evidence is yet available as to whether they possess distinctive structural features. There would appear to be good reason to suppose that these bodies and the transmitting agents are identical.

The interest in these findings does not reside alone in the fact that the bodies of the size of the agents causing two chicken tumors have been demonstrated in the neoplastic cells by electron microscopy but also in the fact that the methods employed offer an opportunity to investigate the relation between tumor viruses and the components of the cells with which they are associated. The method, however, suffers certain limitations in that the extended cultured cells often remain too thick in the central region to permit fine definition. For this reason it has not thus far been possible to ascertain whether the bodies are present in the nucleus or in the region of the centrosphere. Granules, similar to the above, have occasionally been observed in disintegrating and more rarely in apparently intact mitochondria of these tumor cells, but a constant relationship, if any, has not been established by these studies.

As already stated, the tumor agents come down in differential centrifugation with a normal cell constitution of approximately the same size (8), the microsomes, now identified as a component of the basophilic ground substance (9). However, the electron photographs indicate that the two elements have a wholly different appearance. The microsomes, if such they are, appear as hollow spheres or vesicles (Fig. 1), whereas the presumptive tumor agents are dense, rounded bodies (Fig. 2, 3, 4). The growth habit of the chicken tumor agents further emphasizes their apparent independence from the normal constituents of the cell.

As already mentioned, the tumor agents, in order to be seen, must be found in the thinnest parts of the cells. Therefore, in most of the illustrations presented, the agents appear in positions close to the cell surface. In Chicken Tumor 10, the bodies lie side by side in great numbers forming patches of lesser or greater sizes not unlike colonies of staphylococci (Fig. 2). In contrast to this arrangement, the agent of Chicken Tumor I appears scattered and is found singly or at most in groups of 5 or 6 units. Most frequently they occur in pairs, either in close contact or somewhat separated (Figs. 3 and 4) and this happens so often that chance will scarcely account for it. Perhaps the paired entities result from the division of single units, and these are later separated from each other by cytoplasmic currents. If this hypothesis is correct, one might expect the individual bodies to vary in size, becoming larger prior to their division or else much smaller after it. In this connection it should, perhaps, be noted that the diameter of two spheres, one twice the volume of the other, are to each other as 1 is to $\sqrt[3]{2}$; *i.e.*, a difference of only 20 per cent. Differences of this magnitude were encountered in the diameter of the individual bodies present in the cells of Chicken Tumor I a fact already apparent on close inspection of the micrographs.

SUMMARY

1. The cells of Chicken Tumor I and Chicken Tumor 10 spread out so thin when grown in tissue culture that photographs can be taken with the electron microscope giving clear definition of the peripheral portions of the cytoplasm.

[2]The possible effect of certain procedures, notably fixation and drying, on the size and shape of the bodies will have to be taken into consideration in any assessment of the significance of size differences. It should be noted also that the accuracy of calibration of the electron microscope used is probably not better than ± 10 per cent (35).

2. Small bodies, the size of that estimated for the transmitting tumor agents, are present in the chicken tumor cells but are not found in homologous normal cells.

3. The bodies observed in the cells of Chicken Tumor 10 lie side by side in patches often of considerable size, whereas the Chicken Tumor I agent is dispersed singly or else in pairs and only very occasionally in small groups.

4. No evidence has been found to support the view that the bodies are the homologues of normal cytoplasmic constituents or are the products of abnormal cell activities. They have all the appearance of extraneous entities. Their occurrence in pairs in the cells of Chicken Tumor I and their variations in sizes suggest that they may be self-duplicating, possibly autonomous. In all these particulars the chicken tumor agents appear to possess properties commonly attributed to viruses.

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Some Morphological Features of Cultured Rat Sarcoma Cells as Revealed by the Electron Microscope*†

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Several studies of cultured tumor cells have recorded cytological differences between these units and their normal, non-malignant prototypes Thus, Lewis (8, 9) and Ludford (10) have noted in some strains of malignant cells an abnormally large nucleus, showing enlarged nucleoli and increased granularity of the nucleoplasm. But abnormalities of the cytoplasm have been more consistently recorded. In a report of observations on the cytology of living, cultured cells of Mouse Sarcoma 180, Lewis and Gey (7) described the cytoplasm as being "unusually dense." Fell and Andrews (5), using dark-field illumination in a study of cultured cells of the Jensen rat sarcoma, observed the mitochondria to be "finer and less refractile than those of normal mesenchyme cells" similarly examined. From a study of the cells of a large number of rat and mouse sarcomas, Lewis (8, 9) concluded that it is characteristic of tumor cells to show smaller and more numerous mitochondria and a more dense and granular cytoplasm. Carrel and Ebeling (1), though finding no other differences, described the cytoplasm of cells of the Jensen rat sarcoma as being abnormally "coarse" and "refringent." Using dark-field illumination, which is more effective in showing submicroscopic granules, Ludford (10, 11) demonstrated that the cytoplasm of tumor cells "is more finely granular than that of normal cells." Several other characteristics of cultured tumor cells, such as the occurrence of abnormal mitoses, and abnormal reactions to vital dyes, have of course, been noted, but do not fall within the interests of this report.

The cytoplasm of the cultured tumor cell seems, therefore, to contain an abundance of some component which adds to its "density" but which is either too small or not of a proper character to be clearly resolved by transmitted light. This observation and other considerations have made it desirable to examine material of this nature with the much greater resolving powers of the electron microscope.

The initial observations reported in this paper provide evidence to indicate that there are some submicroscopic granular components of the cytoplasm that, in the malignant cells examined, occur consistently in greater numbers and in abnormal form. So far the study has been largely exploratory and subsequent experiments with the same and similar material will seek to confirm and interpret these preliminary findings.

MATERIALS AND METHODS

The cells for these cytological studies have been derived from three rat sarcomas. Two of these (known as 304 and 310) were spindle-cell sarcomas induced in the rat by subcutaneous injection of methylcholanthrene.[1] A third was a Cysticercus sarcoma of the rat (known as I.R.S. 4337) induced by larvae of *Taenia crassicallis*.[2] In addition, a few micrographs have been taken of cultured cells from Mouse Sarcoma 180.

Normal cells, examined as controls, were derived from adult tissues likely to provide cell types similar to those from which the tumors originated.

The cultures of these tissues were generally set up in roller tube flasks, as described in an earlier report (13). Small explants of the tissue were placed in shallow clots of a nutrient-chick plasma mixture (1:1). These clots were formed on small glass inserts that had been previously coated with a thin film of plastic (Formvar). After a few hours of incubation such cultures were fed 0.5 to 1.0 cc. of a nutrient solution. For some experiments this was composed of 4 parts of Tyrode's solution

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** Finney-Howell Fellow.

[1] These were generously supplied by W. H. Lewis. They had been induced approximately two years before these studies were started. Histologically they are similar. Transfers in Wistar strain rats are made approximately every two weeks.

[2] This sarcoma was provided through the kindness of M. R. Curtis. It is carried in the Copenhagen strain and is transferred every 2 to 3 weeks.

(Earle's modification), 4 parts of horse serum and 2 parts of embryo extract. In more recent experiments it has contained 5 parts of Tyrode's solution, 3 parts of human placental-cord serum, and 2 parts of embryo extract. The cultures were fed twice weekly as long as they were continued. When a satisfactory population of cells had grown or migrated from the explant, or when, as for some experiments, a certain period of time had elapsed, the cells were prepared for electron microscopy. Similar cultures were usually fixed and stained for light microscopy.

It has been shown (13) that the cultured cells suitable for electron microscopy are those that spread out on the surface supporting the culture. If a clot is present, the cells that can be used are the ones that have grown out under it. Usually many of these will remain attached to the plastic-coated glass surface when the clot is peeled away. This is done under Tyrode's at a pH between 7.0 and 8.0. The exposed cells are then gently rinsed in a slow stream of the salt solution and finally placed over vapors of OsO_4 for fixation. This is continued for different periods of time depending on the character of the preparation desired. Following fixation the cells, still on the coated glass surface, are washed in H_2O. Portions of the cell population showing the more thinly spread units are selected and the film supporting these is pulled away from the glass and mounted on the fine screen grid commonly used for E.M. preparations. Details of these procedures are described in earlier and current publications (13, 15). The fixed and dried preparations are stored in desiccators over P_2O_5.

An R.C.A. electron microscope, type E.M.U., was used for all microscopy.[3] Most of the micrographs were taken at magnifications between 1,800 to 2,600 diameters and thence enlarged photographically.

OBSERVATIONS

The cells used for electron microscopy were all derived from first generation cultures of the above tumors. In consequence of this, the population probably consisted of more than one cell type. However, as pointed out by Ludford (11), it is characteristic for many tumor cells not to spread as well on a solid surface as their normal prototypes, and this appeared to be true of these sarcoma cells. As a peculiarity of the malignant unit, it could be used in distinguishing them from normal cells. For electron microscope studies the tendency of these cells to become round is a disadvantage, because as mentioned above, only thinly spread units are satisfactory. Most of the electron micrographs have, therefore, been taken of the narrow margins of the tumor cells or of extended pseudopodia. In contrast with the cultures of tumor cells the non-malignant control tissue explants usually supplied a collection of well spread fibrocytes and round cells.

Both the culture characteristics and the morphologies of the cells of the methylcholanthrene-induced tumors, 310 and 304, are so similar that separate descriptions are not essential. Within 48 hours of culturing a narrow ring of tumor cells advances from the explant. The margin of growth remains compact with a rather sharply defined, smooth outline. The cells are generally spindle-shaped and have relatively short processes. Sometimes they develop as cords in which the individual units are essentially cuboidal. In contact with the film-coated glass the typical tumor cell is irregular in outline, and shows 2 to 5 pseudopodial attachments to the glass. These pseudopodia show numerous "ruffles" (9). Between the pseudopodia the cell pulls in markedly towards its center.

The culture characteristics of the Cysticercus sarcoma 4337 have been adequately described by Mendelsohn (12). The cell is likewise spindle-shaped with possibly a lesser tendency to become round. In contact with glass many of the cells show a narrow cytoplasmic margin extending around the entire periphery. The contrast with the normal round cell (clasmatocyte) is obvious in that the margin of the tumor cell is much narrower and shows greater numbers of small pseudopodia ("ruffles") extending from its surface.

The control cultures for these tumors show normal fibrocytes with well extended, thin pseudopodia. The cell population in contact with the glass, especially under the explant, frequently contains round cells with broad, well spread margins that are excellent for electron microscopy.

Electron Microscopy

The observations which follow are based on an examination of about 450 electron micrographs. Of these, approximately two-thirds are of cells from tumor tissue, while the other third is of cells from normal tissue similarly cultured. It seems permissible to draw some additional information regarding the cytology of non-malignant cells from other studies in which cells from the tissues of young (1 day-old) rats have been grown and more extensively examined. Since the particular cyto-

[3]The authors are grateful to Dr. R. M. Taylor for permission to use the instrument that belongs to the Laboratories of the International Health Division of the Rockefeller Foundation.

logical features to be described in this report have been observed in the cells from all three rat sarcomas (and Mouse Sarcoma 180), one description will suffice.

In all micrographs of cultured cells fixed with osmium tetroxide there has appeared within the cell a granular component which is apparently part of the endoplasm or ground substance (Figs. 1 to 7). This portion of the cell usually appears in the form of small granules and vesicles. Frequently these units are strung together to form strands (Fig. 1, c, also Figs. 5 and 6). The diameter of these endoplasmic granules ranges usually from 50 to 200 mμ., but the variation may be greater, possibly reflecting variations in the physiological state of the cells. In general, these units are too small to be resolved by the light microscope. There is good evidence that they are not an artifact of fixation (15).

In the micrographs of malignant cells there has appeared a greater quantity of this endoplasmic material, which gives to the cell a greater density. This picture, however, may in part be due to the less extended state of these cells.

In addition to this excess of endoplasmic material, the tumor cells have been characterized by the presence of abnormally large numbers of extremely dense granules (Figs. 2, 3, 7). These appear to be part of the endoplasmic material (15). They are obviously more osmiophilic than the usual granule or vesicle of the endoplasm and probably owe much of their apparent density to this property. Frequently they appear in groups of 2, connected by a strand to form a dumbbell-shaped structure (Figs. 2, 3, 7). In micrographs of chromium-shadowed cells, they show up as rotund bodies of greater thickness (in the dry state) than the other endoplasmic granules. The pairs look as though they are being pinched apart. Like the less osmiophilic endoplasmic units, these dense granules vary tremendously in size. They may be as small as 40 to 50 mμ and as large as 250 mμ. They are distributed without apparent order in the cell and occur in greater numbers where the other endoplasmic material is more abundant (Figs. 2, 7). Occasionally they form strings composed of several units (Figs. 2, 3) and in some cases the identity of the individual granules is almost lost. In this latter form the strands may appear twisted or as loose spirals (Figs. 5, 6). We have not as yet noticed any correlation between the number of these dense granules and the age of the culture. Cells fixed after 2 days in culture show them as clearly as those fixed at the end of 2 weeks. Also, it has not been observed that varying the time of contact with OsO_4 vapors influences their occurrence.

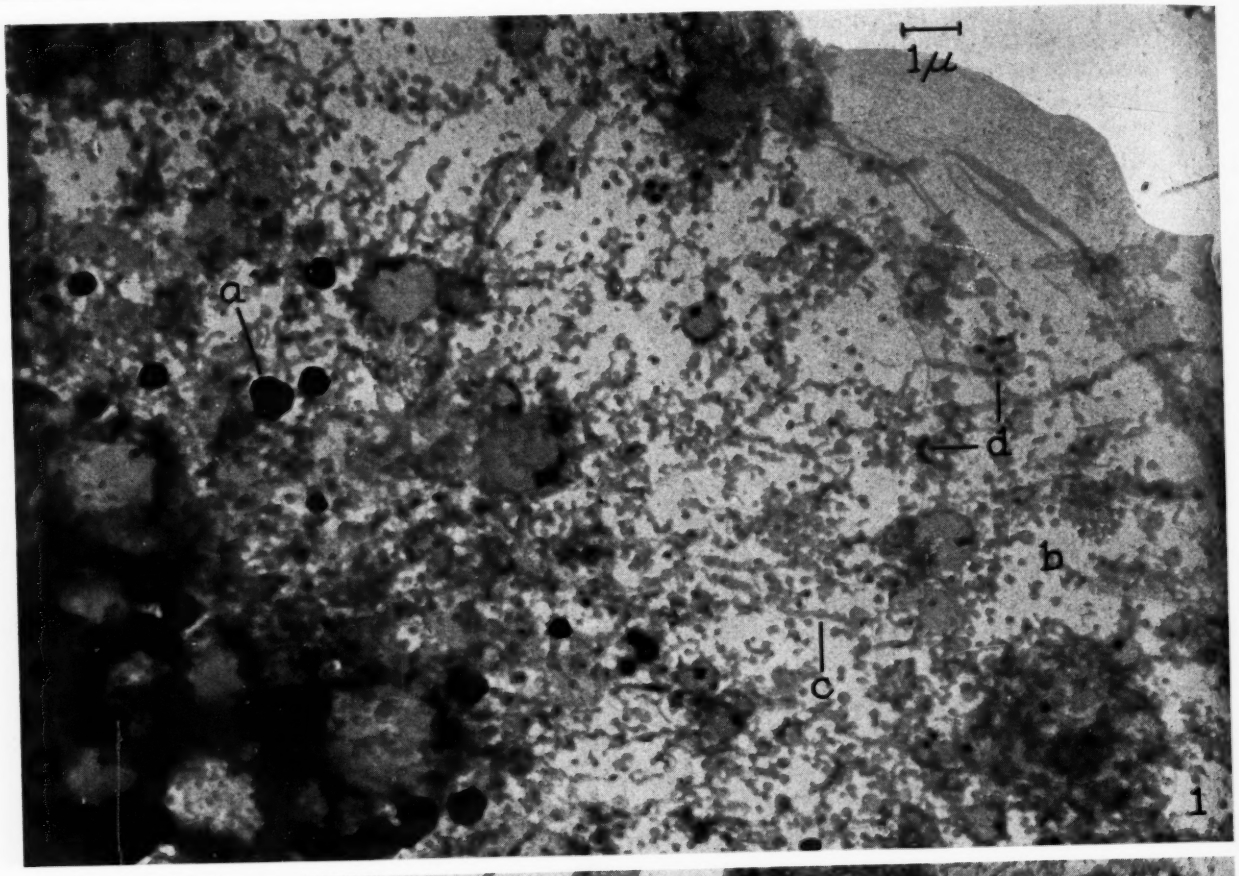
In non-malignant cells, considered as control units, the endoplasmic material has appeared less abundant; likewise the osmiophilic granules are much less in evidence (Figs. 1, 4). In fact, the latter are completely lacking in some micrographs of normal cells. When present, these dense granules show a greater uniformity of size and density (Fig. 4). As in the malignant cells they occasionally form strands which are usually straight (Fig. 4). Instead of the haphazard arrangement, typical of tumor cells, it is characteristic for the strands of the normal unit to be oriented as radii from the central part of the cell.

A more penetrating study of this and additional material will probably reveal other morphological features characteristic of the malignant unit. These that have been described are the more obvious in the limited material thus far studied. It can be recorded that the micrographs also pro-

DESCRIPTION OF FIGURES 1 AND 2

FIG. 1.—Electron micrograph of a marginal portion of a normal rat cell. The edge of the cell is shown in the upper right-hand corner; the center of the cell is out of the picture in the direction of the bottom left-hand corner. The large black units (a) are considered to be Golgi or fat bodies. The small granules and vesicles (b) scattered within the thin margin of the cell are apparently part of the endoplasmic material of the cell. The unit in this material seems to be a small granule about 100 mμ in diameter. But the variation is pronounced and the vesicular form is usually larger. Frequently these are joined together to form strings (c) (not well illustrated in this particular micrograph) (*see* Figs. 4, 5). In this control cell these endoplasmic granules are, with few exceptions, all of the same density. The few of greater density shown here (d) are encountered in much larger numbers in micrographs of malignant cells (Figs. 2, 3, 7). The cell was from a 6 day old culture of an explant of superficial fascia from the inguinal region of an adult rat. It was fixed with vapors of OsO_4 for 24 hours and thereafter washed for 4 hours in H_2O. Mag. × 6,800.

FIG. 2.—Electron micrograph of a marginal portion of a rat sarcoma cell. The border of the cell extends across the upper left-hand corner of the picture. Just within the narrow, transparent margin (a), consisting only of upper and lower cell membranes, is seen the granular endoplasmic material (b). It seems to be more abundant here than in the non-malignant cell (Fig. 1). As part of this there are numerous small, dense, more osmiophilic granules. These vary considerably in size, occur frequently in pairs (dumbbell-shaped) (c) and occasionally form beaded strands (d). Where there is more endoplasmic granular material, as in the thicker parts of the cell (near bottom of micrograph), there are greater numbers of these dense granules. This cell was from a 6 day old culture of a methylcholanthrene-induced rat sarcoma (310). It was fixed with vapors of OsO_4 for 22 hours and thereafter washed for 4 hours in H_2O. Mag. × 6,800.



FIGS. 1-2

vide evidence for a greater number of small pseudopodia on the margins and surfaces of the malignant cell. These take the form of small bulbous protrusions. They are probably the so-called "ruffles" described by Lewis (9).

A small number of micrographs made of cells from Mouse Sarcoma 180 show a large number of dense granules similar in size and form to those encountered in the rat sarcoma cells. On the other hand, micrographs of normal, non-malignant rat and mouse cells derived in most cases from very young animals have shown these dense granules only in rare cases.

DISCUSSION

The electron micrographs of these fixed malignant cells reveal the presence of a minute, dense, osmiophilic granule apparently as a part of the granular endoplasm or ground substance of the cytoplasm. In the tumor cells examined, these granules, along with the less dense endoplasmic material, have consistently appeared in greater quantity and in a form not usually encountered in cells from cultures of normal tissue. It seems reasonable to assume that these features of the malignant cell cytoplasm shown clearly by the electron micrographs account for the conditions of greater "density" and granularity much less clearly resolved by the light microscope (9, 10). It is probable also that the larger of these endoplasmic granules (those larger than 200 mμ) would correspond to the microsomes observed by the early cytologists (*see* Wilson [16], p. 32).

From the frequent arrangement of the dense granules in pairs and in strings, the impression is gained that they multiply and are involved in the production of new endoplasmic material. That is to say, some of these dense granules and strands transform into the granules, vesicles and strands of lesser density that are part of the endoplasm. If they are involved in this way in the formation of new cytoplasm, it is, of course, not surprising to find more of them in the rapidly-proliferating malignant cell. Such an interpretation of their activity suggests a connection between the abundance of these dividing dense bodies and the increased cytoplasmic content of ribose nucleotides reported for tumor cells by Caspersson and Santesson (2). Assuming as seems reasonable, that this endoplasmic material of the electron micrograph is the small particle fraction of broken-up cells, described by Claude (3), this connection with cytoplasmic ribose nucleotides is strengthened by some recent observations that the ribose nucleic acid within rat liver cells is definitely concentrated in the microsomal or small particle fraction isolated by centrifugal methods (6).

The several limitations imposed by the materials and methods of this study make highly speculative, however, any interpretation of the findings, especially in so far as they apply to differences between normal and malignant cells. The observations are based, for the most part, on an examination of only 3 rat sarcomas, 2 of which appear very similar as observed with the light microscope. The effects of culturing and fixation procedures on cytoplasmic components are not well known, although it is difficult to see how the latter of these, at least, could be responsible for the differences shown by the normal and malignant cells. Thus, even if the abnormally large number of dense granules shown by the micrographs of these tumor cells is granted significance, it is not appropriate at this time to describe them as more than another cytological or morphological expression of the malignant state. Future studies will attempt, among other things, to determine the relation of these cytoplasmic components to malignancy and the general phenomena of cell proliferation.

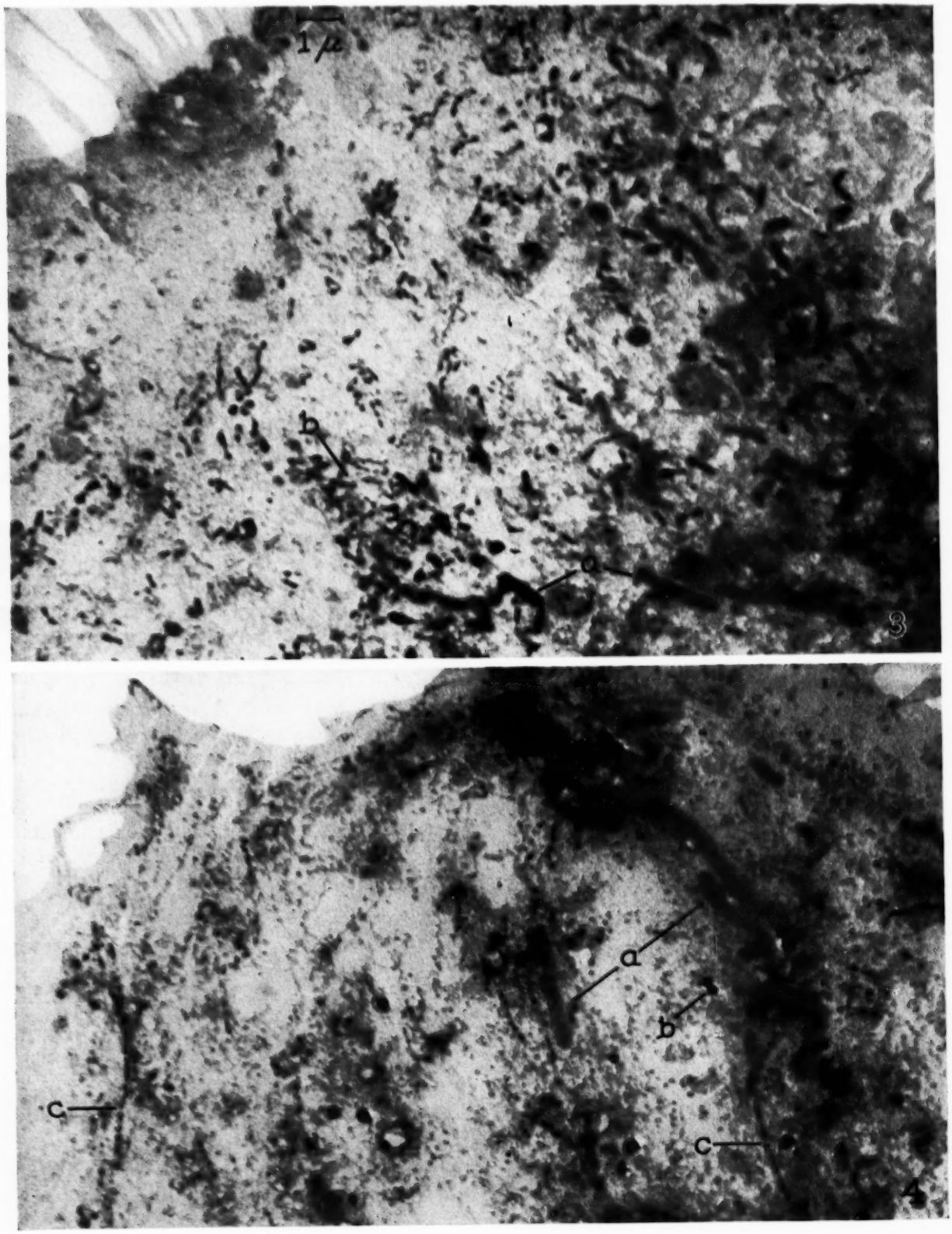
SUMMARY

1. The cytology of cells from 3 rat sarcomas and that of homologous non-malignant cells grown

DESCRIPTION OF FIGURES 3 AND 4

FIG. 3.—Electron micrograph of a part of the margin of a rat sarcoma cell. The edge of the cell, showing the outlines of several small pseudopodia, extends across the upper left-hand corner of the figure. Mitochondria (a) are shown in the more central portion of the cell in the lower right-hand corner of the micrograph. Scattered haphazardly about the cytoplasm are large numbers of the more dense endoplasmic granules (b). It can be noted that they are frequently associated in pairs or in short strings. They vary in size from 50 to 250 mμ. This cell was from a 6 day old culture of a methylcholanthrene-induced rat sarcoma (304). It was fixed with vapors of OsO_4 for 22 hours and then washed for 4 hours in H_2O. Mag. × 6,800.

FIG. 4.—This is an electron micrograph of a small area of the margin of a normal cell. Besides mitochondria (a) and small fat bodies (b), it shows, as part of the endoplasm, a number of small, dense granules believed to be similar to those seen in the micrographs of tumor cells. It is unusual for a normal cell to show as many as this. It is to be noted that they are small (70 to 100 mμ) and, in size, more uniform than those encountered in the tumor cell cytoplasm (Fig. 3). Where they form strands (c) these are straight and directed more or less as radii from the cell's center. The cell was derived from an 8 day old culture of adult rat, subcutaneous connective tissue. It was fixed with vapors of OsO_4 for 24 hours and washed for 12 hours in H_2O. Mag. × 6,800.



FIGS. 3-4

in vitro have been studied with the electron microscope and compared.

2. In the limited material examined, and under the conditions of the study, the malignant cells have been characterized by: (a) a greater quantity of the granular components of the endoplasm; (b) a much greater number of dense endoplasmic granules, which frequently string together in these cells to form spiralling or twisted strands; (c) a greater number of small pseudopodia.

3. It is suggested that these more dense osmiophilic granules are multiplying and thereby producing new endoplasmic material. Their greater number in malignant cells may be related to the rapid proliferation of these units. It is suggested as well that their abundance is linked to the abundance of ribose nucleotides in the cytoplasm of rapidly dividing malignant cells (2).

4. These preliminary observations on only a few malignant units serve mainly to focus attention on these granular components of the cytoplasm and to point the way for further study of similar material by these new methods.

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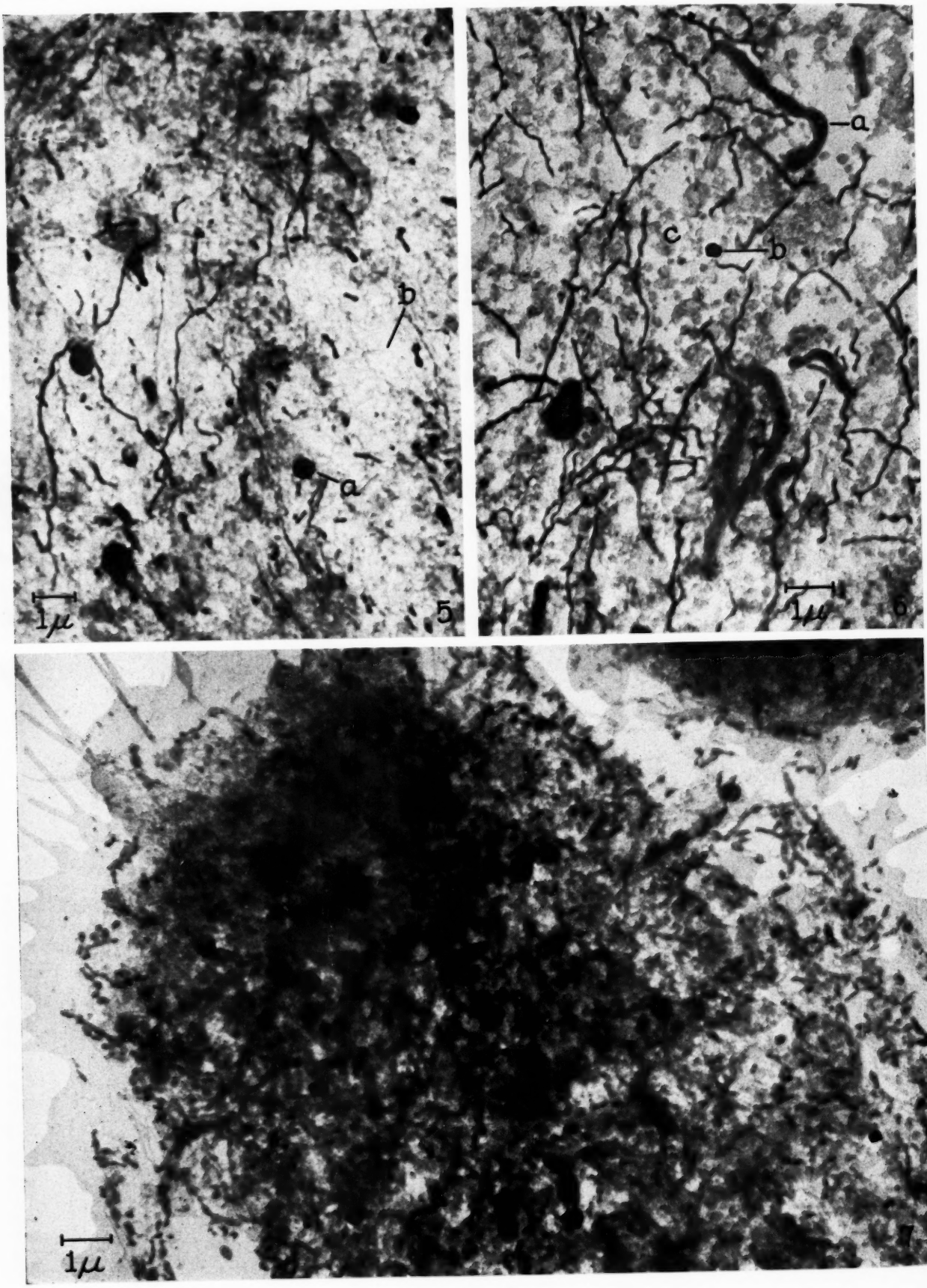
DESCRIPTION OF FIGURES 5 TO 7

Fig. 5.—Electron micrograph of a small area of a thinly spread cell from a culture of a rat sarcoma. It shows the usual osmiophilic fat bodies (a) and a finely reticular endoplasm (b). Mixed with the latter, and apparently part of it, are a number of dense granules (many as dumbbell-shaped pairs) and several slightly twisted or spiralled strands 50 to 150 mμ in width. These latter, which are occasionally encountered in tumor cells, are thought to develop from the pairs and strings of dense granules. Their function is of course unknown. It is possible that they subsequently transform into the less dense fibrillar and vesicular elements of the endoplasm. The cell was from a 6 day old culture of a methylcholanthrene-induced rat sarcoma (310). It was fixed with vapors of OsO_4 for 22 hours and washed in H_2O for 4 hours. Mag. × 6,800.

Fig. 6.—Electron micrograph of a small area of a rat sarcoma cell. There are shown the usual mitochondria (a), fat bodies (b), and endoplasm (c). In addition to these, this micrograph is good for showing an abundance of the twisted and loosely spiralled strands also shown in Fig. 5. It is to be noted that they vary somewhat in length, but in width (100mμ) are surprisingly uniform. They are only one-quarter the width of the mitochondria. It seems to be characteristic for them to have a wavy outline, probably descriptive of a spiral structure. In the original micrograph density variations could be seen along the length of the strands. The cell is from a 7 day old culture of the Cysticercus rat sarcoma (4337). It was fixed 16 hours with vapors of OsO_4 and washed for 3 hours in H_2O. Mag. × 8,000.

Fig. 7.—Electron micrograph of the end of a large pseudopodium of a rat sarcoma cell. The margin of the cell is shown at the left of the micrograph. The several finger-like processes along its edge are illustrative of the numerous small pseudopodia commonly formed by these sarcoma cells (Figs. 2, 3). Within the cell there is a large central mass of endoplasmic material containing a few fat bodies and mitochondria. Dense masses of endoplasm of this sort are frequently encountered near the ends of large pseudopodia. Within the mass, and more clearly shown around its margins are great numbers of the more dense endoplasmic granules. They appear typically in groups of 2 or as short strands. The average width is approximately 100 mμ—or the same as that of the dense strands in Fig. 6. The cell was from a 7 day old culture of the Cysticercus rat sarcoma (4337). It was fixed with vapors of OsO_4 for 16 hours and washed in H_2O for 3 hours. Mag. × 8,400.



FIGS. 5 7

The Role of Sebaceous Glands and Hair Follicles in Epidermal Carcinogenesis

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Squamous cell carcinoma develops more quickly and in a higher percentage of young New Buffalo mice, 2½ to 3 months old at the time of the first painting with methylcholanthrene, than in mice of the same strain 12 to 13 months old (2). In this paper we report the results of treating the skins of still younger mice with methylcholanthrene.

OBSERVATIONS

Individual pregnant Swiss mice were separated and carefully watched for delivery. Thirty young mice, 2 to 10 hours after birth, were subjected to a single application of 0.6 per cent solution of methylcholanthrene in benzene supplied by one stroke of a camel's hair brush, No. 4, extending from the neck down the back to the rump. After 5 months, 1 died; after 7 months, 5 were sacrificed because of body wounds resulting from fighting; and after 10 months, another died. None of these showed any signs of cancer. The remaining 23 mice are now alive and well, 18½ months after this single application of the carcinogen, and show no skin lesions of any kind.

This lack of responsiveness of the skin of newborn mice led to a microscopic study of the skin from the time of birth, or shortly thereafter, until complete development of this structure. Sections of whole skin were examined after treatment with osmic acid, staining with Sudan IV,[1] and coloration with hematoxylin and eosin. Study of the skins of control, untreated, baby mice showed at increasing intervals after birth the following:

10 *Minutes.*—The epidermis well-differentiated, consisting of 5 to 7 strata of cells, covered with a heavy layer of keratin. Sebaceous glands not present, but rudimentary hair follicles visible without hair. (Fig. 1).

30 *Minutes.*—Similar except that a few hair follicles have hair. (Fig. 2).

8 *Hours.*—Epidermis well-differentiated, hair follicles have hair, some of which reaches to the surface of the epidermis. Some small sebaceous glands present. (Fig. 3).

24 *Hours.*—Many sebaceous glands and many follicles with hair in a considerable percentage of cases extending out through the well-differentiated epidermis. (Fig. 4).

72 *Hours.*—Approximately complete development with sebaceous glands attached to every hair follicle, but still not all of the hairs reach out through the epidermis. (Fig. 5).

Adult.—Epidermis only of about 2 cell layers in thickness with very little keratin covering them. Sebaceous glands attached to every hair follicle, and all hairs extending to exterior. (Fig. 6).

Particular attention was paid to the hair follicles and sebaceous glands because of the investigations of Lacassagne and Latarjet (4). These authors have shown that the skin of new born mice (1 day after birth) which was rendered hairless by destruction of the hair follicles and sebaceous glands by ultraviolet radiation, was refractory to the carcinogenic action of methylcholanthrene. On the other hand, a skin which had become repaired after photo-dermatitis and in which some hair follicles and sebaceous glands were restored or were newly formed, gave rise in their experiments

[1]The authors are indebted to Mrs. Virginia Schnettgoeke for developing the staining technic with Sudan IV.

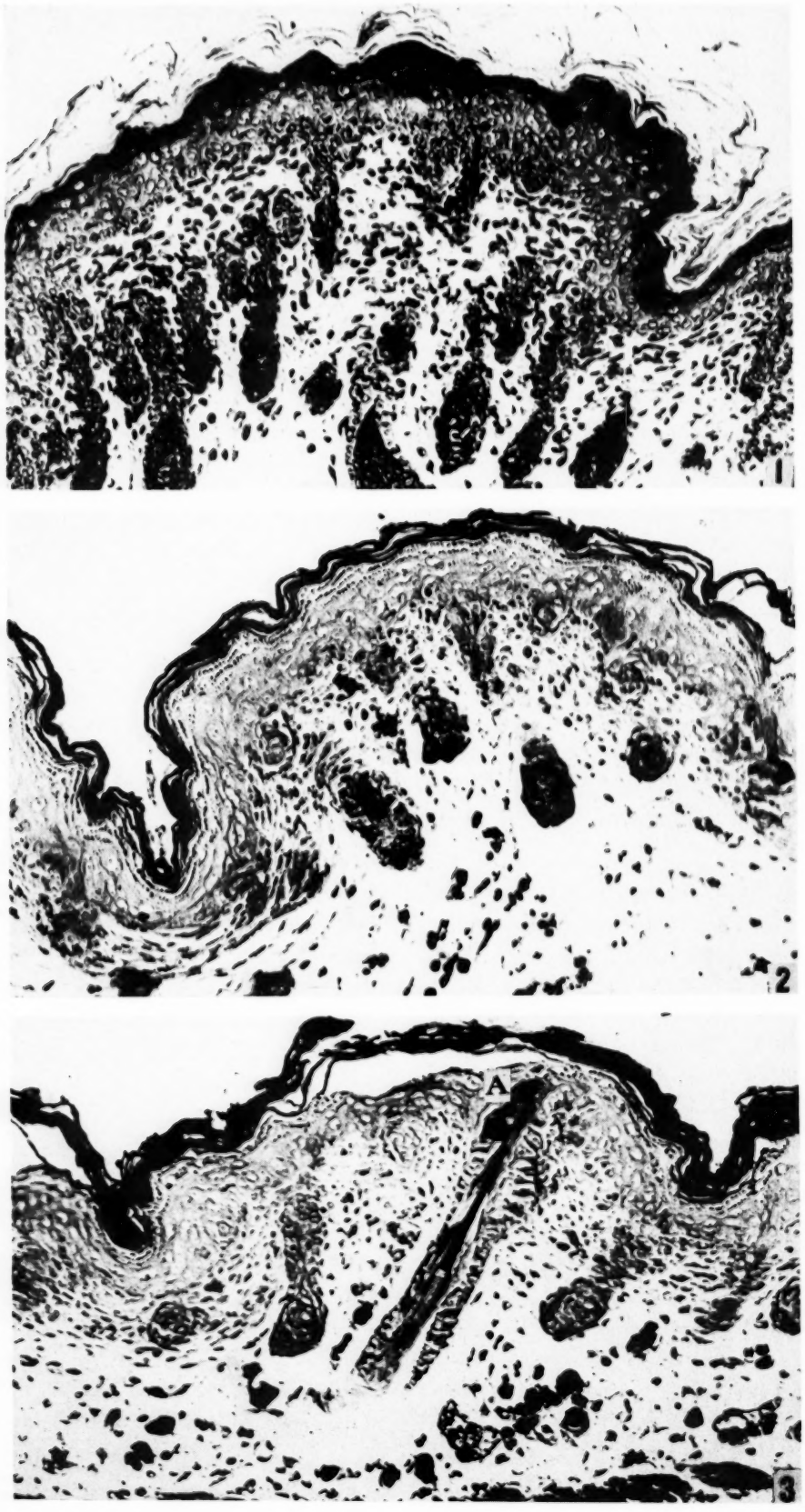
DESCRIPTION OF FIGURES 1 TO 3

All sections Mag. × 193

FIG. 1.—Skin of mouse after birth showing well-differentiated epidermis covered with keratin. There are some rudimentary hair follicles, but no sebaceous glands. Hematoxylin and eosin stain.

FIG. 2.—Skin of mouse 30 minutes after birth. Similar to Fig. 1 except beginning growth of hair in hair follicles. Osmic acid stain.

FIG. 3.—Mouse skin 8 hours after birth revealing thick epidermis, which is covered by much keratin. Some hair of the hair follicles protrudes through the epidermis. Anlage of some sebaceous glands is present, as identified directly below letter "A". Osmic acid stain.



FIGS. 1-3

to rapidly growing cancer after subjection to the same carcinogen. They, therefore, suggested that the hair follicles and sebaceous glands play an important role in skin carcinogenesis.

The experiments of Simpson and Cramer (7, 8) on the distribution of methylcholanthrene after a single application to the skin of mice has led to a better understanding of its mode of action. They found that, immediately after the carcinogen was applied, it localized in the sebaceous glands and in the free lipids of the keratinized epithelium. Four to 5 days later there was degeneration and disappearance of the sebaceous glands, accompanied by a massive excretion of sebum, containing the carcinogen, into the hair follicles and then onto the surface of the epidermis. The fluorescence of the methylcholanthrene in the sebum was blue-violet in contrast to the yellowish-green fluorescence of the unchanged carcinogen on the keratinized surface indicating, perhaps, that the sebaceous glands are in some way concerned in the metabolism of methylcholanthrene.

DISCUSSION

The absence of cancer production by methylcholanthrene in the epidermis of new born mice (2 to 10 hours after birth) may be due to several factors. That it is not due to the amount of carcinogen delivered is evident from the observations of others who have studied the effect of a single application of methylcholanthrene to the skin of mice. Mider and Morton (5, 6) were the first to demonstrate that one treatment with this carcinogen was sufficient to induce epidermal cancer in the C57 brown strain, but not in the C57 black mice. Later on Cramer and Stowell (3) found that about 43 per cent of young Swiss mice (2½ to 3 months of age) developed cancer after a single treatment with the same carcinogen, and Simpson and Cramer (9), likewise, showed that 3 out of 12 young New Buffalo mice responded, by cancer formation, to the carcinogen administered under the same conditions. In the experiments of Cramer and Stowell (3) and of Simpson and Cramer (8), each mouse received 3 strokes of the brush with the carcinogen on a large unepilated area. Since baby mice are hairless, the single stroke given to them in our experiments is comparable to the 3 strokes by these authors to the older mice. Probably our new-born mice received per unit area an amount of the carcinogen equivalent to those painted in the unepilated condition.

How, then, are we to explain the fact that cancer did not develop in the epidermis of the new-born mice? Failure to respond by cancer formation to a dose of carcinogen which appears to have been adequate for older mice may have been due to one or more factors. Their epidermis was thicker than that of older mice and this circumstance may have prevented its entry. Their hair follicles were few in number with only occasional hairs reaching the surface and their sebaceous glands were few, small and incompletely developed, which might result in the portals of entry supplied by these structures for the carcinogen being less open than in older mice.

Mention should be made, also, of similarity between epidermis of new-born mice and epidermis of adult mice repeatedly treated with methylcholanthrene. Both are thick, keratin-coated and deficient in sebaceous glands. It would appear that the sebaceous glands, when present, have a conditioning effect upon the epidermis, and that the decreases in the calcium, iron, copper, zinc, and other constituents in hyperplastic epidermis (1) are definitely associated with their disappearance. The chemical alterations induced throughout the hyperplastic stages are maintained in the absence of these dermal structures. It is apparent, therefore, that the role of sebaceous glands and hair follicles in skin carcinogenesis is a very important one.

SUMMARY

The epidermis of new-born mice (2 to 10 hours after birth) has been found refractory to a single application of methylcholanthrene. The hair follicles and sebaceous glands at this time are still rudimentary, becoming almost completely developed at 72 hours after birth. The importance of sebaceous glands and hair follicles in the development of skin cancer is discussed, and the conclusion is reached that their condition in new-born mice is related to the resistance of such mice to the carcinogenic effect of methylcholanthrene.

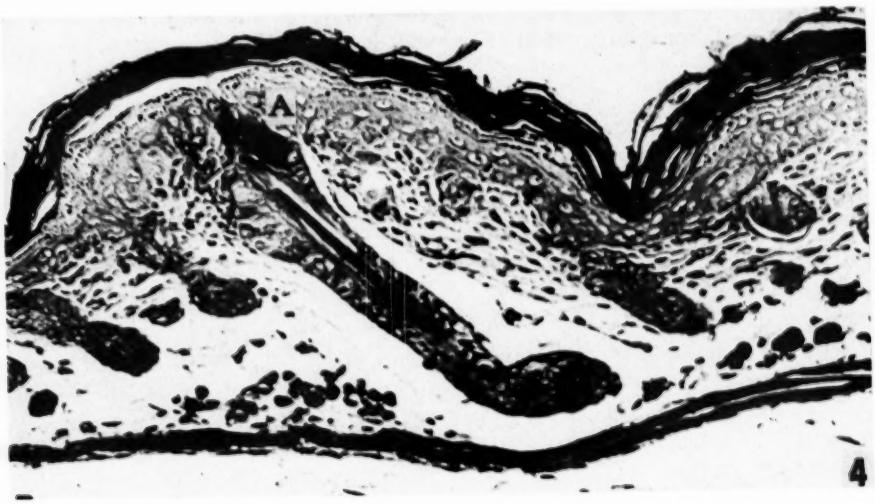
DESCRIPTION OF FIGURES 4 TO 6

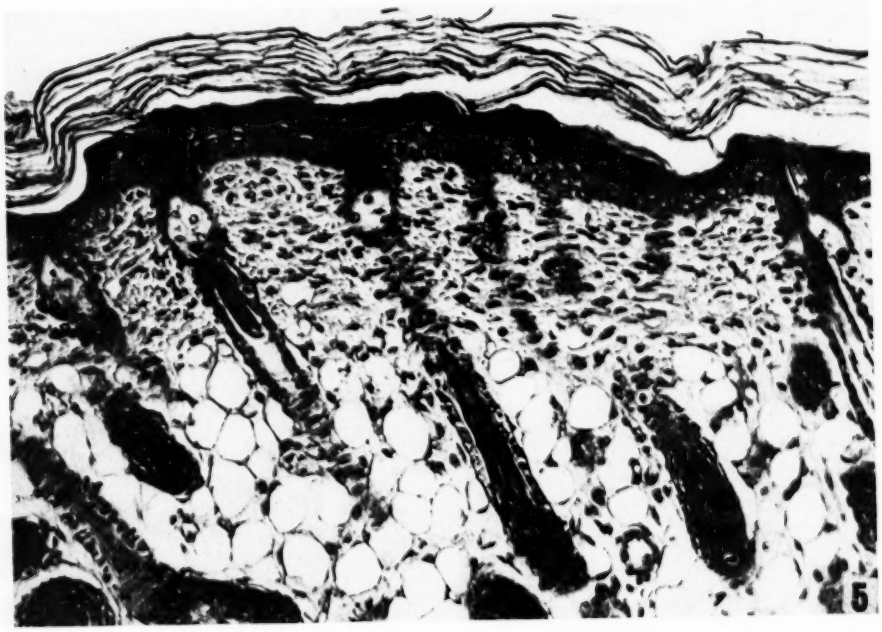
All sections Mag. × 193

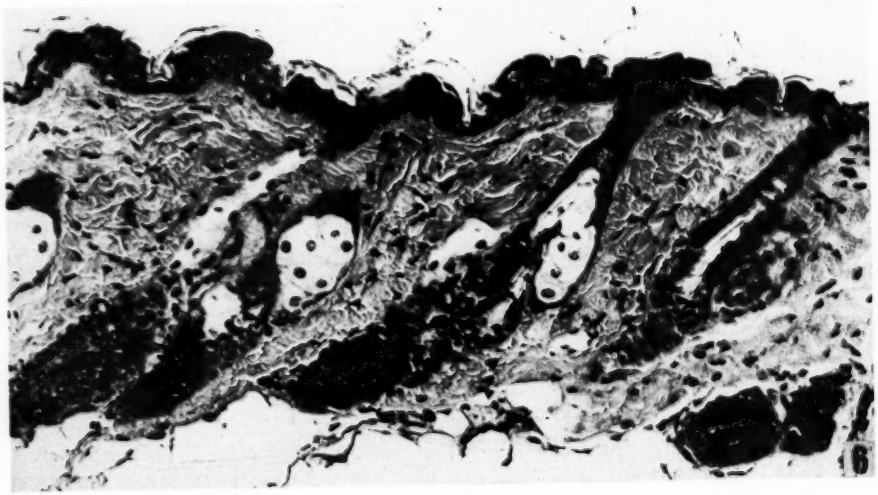
FIG.4.—Skin of mouse 24 hours after birth with epidermis consisting of layers of cells which are covered by much keratin. Some hair has protruded through the epidermis and many sebaceous glands are appearing directly below letter "A." Osmic acid stain.

FIG. 5.—Skin of mouse 72 hours after birth showing sebaceous glands associated with every hair follicle. Hematoxylin and eosin stain.

FIG. 6.—Epidermis with little keratin of skin of mouse 3 months of age. Sebaceous glands and hair follicles are completely developed. Hematoxylin and eosin stain.







FIGS. 4-6

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The Carcinogenic Activity of 2-Acetaminofluorene

II. Effects of Concentration and of Duration of Exposure

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The production of carcinoma in rats fed diets containing 2-acetaminofluorene has been demonstrated by Wilson, DeEds and Cox (8). Albino rats were fed diets containing 0.031 per cent or more of acetaminofluorene (AAF) for periods ranging from 95 to 333 days. Growth was retarded, and hyperplastic and frequently carcinomatous lesions of a variety of tissues developed in all of the animals living for a long enough time. Bielschowsky and Green (4) fed AAF to rats and confirmed the effect on growth. Their data were expressed as mgm. of AAF per day, while we reported the AAF concentration in the diet. Considering average amounts of food ordinarily eaten by rats, it is probable that the growth effects were comparable in the two studies. Bielschowsky (2) later extended this work and in 93 rats found 105 malignant tumors. This work was very much in agreement with ours except that in the former no tumors of the urinary tract were found, and there were several cancers of the intestine, while we found that lesions of the urinary tract were frequent, but noted only one carcinoma of the colon. Armstrong and Bonser (1) gave AAF to mice of the CBA strain (mammary cancer-resistant); of the 12 animals, 10 survived for 32 or more weeks, and of these, 8 developed tumors. Five primary tumors, 4 of them malignant, were found in the urinary bladder. There were 5 tumors, 1 with malignant areas, in the livers, and 2 tumors, 1 malignant (sarcoma) in the uterus. There were no tumors of the ductus acusticus or breast, although a mild breast proliferation was noted. Neither Bielschowsky (3) nor Wilson, DeEds and Cox found any unusual growth in the thyroid, but if the animals were simultaneously treated with allylthiourea to obtain a goitrous gland, AAF led to benign and malignant tumors of the thyroid.

The experimental work on which this paper is based was completed in 1942, but because of the pressure of other work the authors were unable to prepare their results for publication.

As a consequence of the work reported in the first paper (8), further experiments were started to find out more details of the carcinogenic activity of AAF. Due to war-time interruption of the work, assembling of the data was delayed. This paper presents the work on the concentration of, and the duration of exposure to, AAF required to produce neoplastic changes. A comparison of the actions on rats and on mice is made. A generalized discussion of the microscopic appearance of tissue of animals in this and other papers is presented in a separate report (5).

EXPERIMENTAL

Rats.—The colony of rats was the same as that described previously (8). When some difficulty was experienced in obtaining effective transplants of tumor tissue, all breeding was changed to brother-sister matings. Some of the later animals were from this more highly inbred strain. The AAF was from the same batch used previously, and was furnished by the Insecticide Division of the Bureau of Entomology and Plant Quarantine, U. S. Dept. of Agriculture (8). The diet was the same as before. AAF was mixed with the diet in the desired concentrations and fed to the animals *ad libitum*. At approximately weekly intervals the rats were examined carefully. Ordinarily the animals were killed and autopsied when their condition became serious; a few died during the night and were autopsied the next day unless the viscera had been eaten by the other rats. Tissues were fixed in 4 per cent formaldehyde solution, and paraffin sections were stained with hematoxylin and eosin for microscopic examination.

Effect of concentration.—In the first paper (8) we presented data on rats receiving diets containing 0.031, 0.062 and 0.125 per cent AAF in the diet. All these animals, if they lived long enough, developed tumors, and the effectiveness was about the same on the lowest concentration as on the highest. It was considered desirable to find out how low a concentration of AAF would produce

tumors. The data have been extended to diets containing 0.016, 0.008, 0.004 and 0.001 per cent of AAF. There were 6 animals on each of these diets except the lowest, where there were 5. The animals were males, except for those on the 0.004 per cent diet. Tumors like those previously seen developed in all the rats on the 0.016 and 0.008 per cent concentrations. Five of the six rats on the 0.004 per cent diet had similar gross changes, although microscopically, incidence and degree of associated nodular hyperplasia were smaller than in animals fed large quantities of the compound. None of those on the 0.001 per cent AAF diet had lesions which resembled the usual cancerous tissues from animals on higher concentrations.

Histological sections were made of the heart, lung, liver, spleen, kidney, adrenal, testis, bladder, pancreas and thyroid of each of the 5 rats fed 0.001 per cent of the compound for 741 days. There was atrophy of some of the organs, probably explained by the advanced age of the animals when they were killed. Several had chronic inflammatory changes in the lungs, and one showed bronchial epithelial metaplasia to stratified squamous cells, but this did not suggest a specific effect. Similarly chronic inflammation of the pancreas in 2 animals could not be related to the treatment. No distinct hyperplastic nodules of liver cells or cysts were seen in any of the animals, and the bladders were normal in all. One or both of these organs have been altered in practically all animals receiving as little as 0.004 per cent of AAF in the diet. Therefore, the significance of a single nodule composed of fibrous tissue and bone in the liver of one animal and of a small nodule of fibrous tissue and blood vessels resembling an angioma in the spleen of another animal is questionable. These were the only tumor-like lesions in this group of animals. Even assuming that AAF influenced the development of these 2 nodules, which were different from any tumors developing in the other experimental animals, it must be concluded that the effect of this quantity of the material is at most minimal.

The effect of the concentration of the compound in the diet is shown not only by the incidence of tumors, but also by the length of time the rats could be kept on the diet before the tumors necessitated killing the animals. The average time on the diet for the rats receiving the 0.125, 0.031 and 0.016 per cent diets were, respectively, 211, 313 and 305 days. A concentration of 0.008 per cent allowed the animals to live for an average of 407 days; the time was 645 days for the 0.004 per cent diet, and the animals on the 0.001 per cent diet were sacrificed without signs of tumor after 741 days.

Duration of administration.—In the previous report (8) it was mentioned that 5 rats that had received AAF in their diet for 95 days and then were placed on control diet subsequently developed cancer. It was stated also that a single, large gastric administration of the compound did not seem to be carcinogenic. The 4 animals still alive when that report was written were autopsied from 15 to 22 months after the AAF was administered. One had a mammary adenoma in the groin and some cortical nodules in one adrenal. Some of the others had cystic ovaries or pneumonia, neither of which seemed to be attributable to the AAF.

Since 95 days' exposure will produce tumor and 1 day's will not, the question arises as to how long the animals must eat the substance to initiate those changes which will ultimately develop into malignancy. The periods of exposure selected were 25, 50, 75, 100, and 150 days. The concentration of AAF at the start was 0.125 per cent. Because of the poor physical condition of the animals still on the experimental diet, the concentration was decreased after 54 days to 0.031 per cent. All animals were returned to the control diet at the conclusion of the respective feeding periods. Each group consisted of 9 to 12 animals with approximately equal numbers of each sex. With the exception of 1 rat, which died of pneumonia 112 days after the beginning of the experiment, all of the animals receiving AAF for 75 or more days developed macroscopic tumors. Microscopic examination regularly showed characteristic changes in the tissues of 15 of the 16 rats examined, the 16th being the rat that died of pneumonia.

Of the 11 rats in the 50 day group, 6 had gross tumors, 3 had lesions of questionable character, and 2 were grossly normal. Tissue sections from 10 of these animals were studied. Less frequent alteration was found than in the rats receiving the diet for a longer time, but every animal showed some change. All the livers contained nodules of hyperplastic hepatic cells or cysts, although only 2 bladders showed definite hyperplastic changes. The bladder of a third animal had a grossly visible nodule but this was not included in the section. There were 3 mammary adenomas, 2 subcutaneous fibromas, 1 gross nodule suggesting mammary tumor which was not sectioned, 1 adenocarcinoma of the colon with metastases, 1 papillary carcinoma of the renal pelvis, 1 adenoma of kidney tubules, and 1 unclassified malignant tumor of the mediastinum suggesting origin from the thymus gland. Three rats from this group had lung tumors; 1 was

a small-cell carcinoma surrounding a bronchus, and the others were nodules in which the aveoli were almost filled by proliferated lining cuboidal cells. In the 2 uteri from the animals examined there was local proliferation of endometrial glands.

Twenty-five days' feeding of the compound led to 6 animals with gross tumors, 1 with possible tumor, and 4 with grossly normal tissues. Microscopic examination disclosed occasional changes like those in the other treated animals. They were less frequent and in most animals were minimal. The 9 livers studied histologically exhibited occasional small cysts and 3 showed distinct nodules of hepatic cells. Two female rats showed local proliferation of glands beneath the endometrium similar to those previously described. Only 1 ovary was sectioned from this group; there was a serosal cyst, and in addition, there was marked swelling of the ovarian stromal cells. No follicles were seen in the section. There were 2 mammary fibroadenomas in 1 animal. Two bladders from this group contained papillomas; one spleen showed a fibrous nodule, apparently a fibroma. Two lungs showed focal proliferation of alveolar epithelium like those described in the previous group. A period of 25 days seems to be an approach to the lower limit of exposure necessary for carcinogenic action. Although nodular hyperplasia and neoplasia were not as frequent in this group of animals as in those receiving AAF for a longer time, there was nevertheless a definite carcinogenic effect.

Time of exposure as well as concentration of the agent, is a factor in determining the time required for development of the lesions. The groups receiving AAF for 75 or more days developed tumors in about equal times. The average for these groups from beginning of experimental feeding to the time of autopsy varied from 227 to 295 days. For a 50 day feeding period, the interval increased to 406 days, and for the 25 day period, to 554 days.

In the AAF feeding experiments to determine the minimum effective concentration and the shortest time of exposure, 108 rats were used—53 males and 55 females. Microscopic examination was not made on all animals, so the following figures cannot be interpreted to mean number of malignancies, since some malignant lesions were observed which were not apparent grossly and some large tumors were found to be benign. The principal tumors noted on gross examination were: 44 per cent of the animals had tumors of the bladder, and 7 per cent had renal tumors. The incidence and time of development of these 2 types of lesions were about the same for both sexes. Strikingly abnormal livers were found in 20 per cent of the females and in 55 per cent of the males, a little earlier in the males than in the females. The average time for males was 317 days from the start of feeding of AAF; for females it was 411 days. Tumors of the mammary type were noted in 34 per cent of the females and 8 per cent of the males, the time of development being about the same for both sexes. The subcutaneous tumors of the head were found in 29 per cent of the females, 17 per cent of the males, and with equal time intervals. Apparently the liver of the male rat is more susceptible than that of the female, whereas the opposite is true for the head and the mammary type of tumor. Bielschowsky (2) gave the incidence of malignant tumors in his rats; he also found a sex difference for liver, mammary gland and head. The greatest difference between Bielschowsky's results and ours is the complete absence of tumors of the urinary tract in his animals.

An impression is obtained, when conducting experiments like those just considered, that the mammary, and particularly the head, tumors develop earlier than do tumors of the internal organs. This impression is not clearly borne out by the assembled data. Obviously, the external tumors are more easily noted than are those of the internal organs, and they ordinarily appear to grow more rapidly, and so, being more spectacular, are more quickly recognized.

Mice.—Since this colony of rats had not been used for cancer studies previous to the work reported earlier (8), it was considered advisable to check the results by using some of the strains of mice frequently employed for carcinogenic experiments.[1] Five levels of AAF-containing diets were fed to C57 mice. The AAF concentration varied from 0.031 to 0.5 per cent of the diet. Four females and 2 to 4 males were started on each level. Liver, bladder or kidney lesions, grossly similar to those developing in the rats, were found in most (11 of the 16 mice alive at the time the first tumor was discovered) of those receiving 0.125 per cent or more of AAF and in some of those receiving the 0.062 per cent diet. Pathological changes not clearly tumorous were found in the mice given the lowest concentration of AAF. The first tumors did not appear quite as early in mice as in rats, and the experiment had to be terminated sooner because of the poor condition of the remaining mice. The average length of time from the start of the AAF diet to autopsy was 349 days.

[1]We are indebted to Dr. John F. Menke of Stanford University School of Medicine for furnishing us with these mice.

The histological findings were more specific. Of the mice that received 0.5 per cent of the substance in the diet only the 1 animal surviving as long as 248 days showed any specific tissue change, and this was a moderate nodular epithelial proliferation in the liver. The 2 animals that lived less than 250 days on the 0.25 per cent diet showed no liver changes; however, 1 had a carcinoma of the bladder. The 4 mice that lived longer showed proliferated nodules of hepatic epithelial cells, although in one animal the proliferated structures were only small ducts. Three of the livers showed distinct adenomas. Three other bladders from this group showed irregular epithelial proliferation. One of these was carcinomatous. One animal had a mass of chronic inflammatory tissue in the perinephric region but no tumor tissue could be identified here.

Of the group receiving 0.125 per cent of AAF in the diet, 1 animal died of leukemia in less than 100 days. It had no other lesions attributable to the treatment, although prominent epithelial cells were seen lining pulmonary alveoli throughout most of the lung section. Another animal died in 228 days with no characteristic lesions. The remaining two animals in this group as well as the 3 receiving 0.062 per cent AAF, all lived 248 days or more, and all showed proliferative epithelial lesions. All of the livers showed epithelial nodules of hepatic cells, the extent of which bore no apparent relation to the length of life. Two were classed as adenomas. Two animals showed focal, non-malignant proliferation of the bladder epithelium, whereas in 2 others there was a questionable epithelial thickening of the bladder. One animal had an unexplained hydronephrosis.

Of the 8 mice which received 0.031 per cent AAF in the diet, 6 showed no recognizable epithelial proliferation of the liver, and in the other 2 there were only questionable changes. Two had irregular, non-malignant epithelial proliferation in the bladder, and one showed early bladder carcinoma. One animal had cellular infiltrations in the kidney and lymph nodes suggesting leukemia. Another showed an aggregate of small glands with a small cyst at one side of the endometrial lumen, similar to the lesions described for the rat uterus. This was 1 of only 2 mice from which the uterus was examined histologically. One mouse had a chronic skin ulcer without any evidence of tumor.

Three male C3H mice given 0.125 per cent AAF for 294 to 372 days were autopsied. In these 3 mice there were 2 instances of nodular hyperplasia of liver cells with adenoma formation, 2 bladder carcinomas, 2 instances of slight irregularity in the size of the pancreatic acini, 2 of unexplained hydronephrosis and 1 pulmonary nodule in which the alveoli were partly collapsed and lined by cuboidal epithelial cells. This was similar to the benign pulmonary nodules in several of the rats.

Twenty mice of the Bagg albino strain, half males and half females, were kept on diets containing 0.062 or 0.125 per cent AAF until death or to the end of the experiment, which lasted 322 days. There was no striking difference between the animals on the 2 concentrations. Seven of the animals developed grossly visible tumors, 7 more had other grossly visible lesions. Two of the females had mammary tumors. Six of these animals were examined histologically. Only 2 animals showed recognizable nodular proliferation in the liver. Chronic cystitis was present in all, but only 2 showed distinct bladder epithelial proliferation, and 1 of these had early carcinoma of the bladder. Four of the mice showed slight irregularity of pancreatic acini, the significance of which is uncertain. Two had subcutaneous carcinomas, probably of mammary origin, and one of these had nodular hyperplasia with cyst formation in another sectioned mammary gland. In 1 animal from which sections of the uterus were studied there was irregularity in the endometrial glands, some of which were cystic.

Transplantation of tumors.—Several attempts to transplant the AAF-produced tumors into rats were made. When the parentage of the recipient differed from that of the donor, only 2 of 22 into which transplants were made gave a positive response. However, when the parentage of the animals was the same, 6 of 12 recipients developed positive tumors. Of 12 transplants which were examined histologically, 7 showed abnormal tissue growth suggesting tumor, and one, after secondary transplantation, showed a cyst lined by folded columnar epithelium. In most cases the growth at the site of the transplantation was similar to that of the primary lesion, although 1 animal into which an adenocarcinoma was transplanted developed a sarcoma composed of bundles of closely packed, rather small spindle cells. Of particular interest is the observation that two transplants in this animal grew and sections of both transplanted tumors had the same structure. It may be of significance that the peripheral portion of the primary tumor, from which the transplants were obtained, showed unusual amounts of very cellular fibrous stroma containing mitotic figures. This phenomenon of sarcomatous growth following transplantation of epithelial tumors has been described in transplantable spontaneous mammary tumors of mice (*see* reference 6, page 29). The specific tumors from which secondary growths

were obtained on transplantation were: squamous cell carcinoma of the head, subcutaneous tumors probably of mammary origin, and liver tumors. One of each of these types was retransplanted, satisfactorily in each case, at least in so far as gross appearance indicated, and there was microscopic verification in the case of the head tumor transplant.

Twenty-one C57 mice received transplants and growths developed in 18 of them. Gross observation indicated successful transplantation from bladder, kidney and liver, and one bladder tumor transplant was satisfactorily retransplanted. Histological study showed successful transplantion of bladder and liver carcinomas; no kidney tumor transplants were examined microscopically. There was one case of leukemia from which lymph node lesions were transplanted and retransplanted successfully.

DISCUSSION

The carcinogenic action of AAF on rats has been found to be present when the concentration in the diet was as low as 0.004 per cent, and, with higher concentrations, the material did not have to be administered for longer than 25 days. It is interesting, although perhaps accidental, that the amount of AAF ingested by the animals eating for the minimum time and by those eating a minimum concentration, was about the same. Assuming that each rat ate 10 gm. of food a day, a rat on the diet for 25 days would ingest about 0.3 gm. of AAF, and one on the 0.004 per cent diet about 0.25 gm. However it must be remembered that 1 gm. in a single dose was not effective in producing carcinoma.

Mice were apparently somewhat more resistant to the action of AAF than were rats. They tolerated a higher percentage of the substance in the diet, fewer lesions appeared than in rats given the same dose, and the time of appearance was longer than in rats fed moderate doses. However, the types of tissue change were similar to those seen in rats, and many distinct tumors were present. Some were malignant as judged by the invasive property of the abnormal cells and by transplantation experiments. No metastatic tumors were recognized in mice. Most of the organs frequently affected in the rats were also the seat of nodular epithelial hyperplasia and tumor formation in the mice, but the relative frequency and severity of the changes was different in the two species. The most striking differences were the complete absence of squamous cell tumors of the side of the head in mice, absence of distinct metaplasia of the epithelium of the renal pelvis while bladder tumors were common, and the much smaller incidence of mammary tumors in this species.

The presence or absence of mammary tumors warrants further consideration. In our rats, especially females, breast tumors were quite common. In the mice, only 2 of the female Bagg albinos developed tumors and none of the C57 animals. Armstrong and Bonser (1) found no mammary tumors in their mice, but there was a mild breast proliferation. Bielschowsky (2) discussed this question in some detail. He found no estrogenic activity induced by AAF, and he stated that if a highly estrogenic substance were responsible for the mammary tumors, they should be found with equal frequency in male and female rats. However, he discovered, as we did, that there were many more tumors in females than in males. Novelli and Giunti (7) have reported that certain derivatives of fluorene have a weak estrogenic activity in rats. They did not study any aminofluorene compounds. In a preliminary experiment we were unable to find any estrogenic effect with AAF; however, the rats were in poor nutritional condition and this could have influenced the results. One of us (A. J. C.) later observed normal estrual cycles in several rats on AAF. This will be reported in greater detail later.

In the first paper on AAF (8) it was stated that occasionally in older breeding females of this colony, there has appeared a large, localized mammary tumor. These have been found to be benign adenomas or fibroadenomas. The number of such tumors has now reached a total of 14, from 500 breeding females. One unclassifiable malignant tumor with numerous metastases was found in a female rat well over 2 years of age. This animal was a laboratory pet and her diet was unconventional, but in no case was she subjected to any experimental diets or procedures. No spontaneous tumors have been seen in males.

Our observations suggest that there may be a relationship between the frequency of development of mammary tumors after AAF feeding and the incidence of spontaneous tumors of the mammary gland. In the few strains of animals studied, epidermoid tumors of the head occurred only in those animals (rats) that showed frequent mammary tumors.

SUMMARY

1. 2-Acetaminofluorene (AAF) had carcinogenic action on rats when its concentration in the diet was as low as 0.004 per cent. A diet containing 0.001 per cent had no recognizable carcinogenic effect.

2. When the concentration in the diet was

0.125 per cent, 25 days feeding was sufficient to initiate changes which showed up some time later as cancers. A single large dose into the stomach was not carcinogenic.

3. As the concentration of AAF decreased, or the time of administration became shorter, there was an increased interval before tumors were observed.

4. Tumors like those produced in rats were observed in 3 strains of mice. The mice were more resistant than the rats as judged by frequency of tumors, time for tumor development, and concentration of AAF which the animals could tolerate. Mammary tumors in C57 mice were absent, and infrequent in the Bagg albino strain; epidermoid tumors of the head were not seen in any of the mice, although they were frequent in the rats.

ACKNOWLEDGMENT

The authors wish to thank Mr. E. K. Doxtader for his painstaking care in the preparation of the many tissue sections.

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The Carcinogenic Activity of 2-Acetaminofluorene

III. Manner of Administration, Age of Animals, and Type of Diet

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Wilson, DeEds, and Cox (4) have shown that 2-acetaminofluorene (AAF), when incorporated into the diet and fed to rats for some time, produced hyperplastic and carcinomatous lesions in the animals. At the time that report was written, certain animals that had received the compound by various other routes of administration were still alive and well. The authors drew the tentative conclusion that "the failure to produce tumors in the parenterally treated rats [suggests] that continued administration by way of the gastrointestinal tract is necessary for carcinogenic activity." The preliminary work on this phase of the subject was continued and expanded, and the results form the subject matter for this paper.

EXPERIMENTAL

Single oral dose of AAF.[1]—A single, large dose of AAF by mouth does not lead to tumor formation. This experiment has been reported in some detail previously (5) and it need not be enlarged upon here. The important point is that AAF is carcinogenic when given by mouth only when the administration is continued for a period approaching 25 days. Longer administration increases the incidence of tumors and decreases the time required for initiation of the lesions.

Subcutaneous implantation of crystalline AAF.—A previous report (4) told of the implantation of approximately 0.5 gm. of crystalline AAF in the subcutaneous tissue of the groin of 5 female rats. (By mistake they were described as males in the earlier paper). After 14 months there were no gross signs of tumor. The animals finally were autopsied 504 to 728 days after the date of implantation. Two of them developed subcutaneous tumors in the mammary region, one had several growths in the uterus, and all 5 had a few small cystic areas in the liver suggesting mild chronic changes due to AAF. Microscopical examination of the tissues revealed that all showed changes like those seen when the compound was administered by mouth. The extent of the changes was about that of the group which received 0.125 per cent in the diet for 25 days (5). Four of the livers showed small cysts. Two of 3 ovaries examined showed proliferation of abnormal stromal cells, and one contained a cyst lined by a single layer of partly ciliated cuboidal epithelial cells. One animal had a carcinoma of the lung composed of rather small epithelial cells in a dense fibrous stroma. Some of the cells were in small clusters but no gland formation was seen. The same animal had a well differentiated adenoma of the uterus. The other animal from which the uterus was examined showed marked nodular proliferation of small glands beneath the endometrium. One animal had 5 subcutaneous nodules, one at the site of implantation. As the section of the latter was not specifically indicated, it is not certain which of the 5 nodules in the sections represented this lesion. However, 3 were adenomas, probably of mammary origin, one was a hyperplastic mammary gland; the fifth, a fibroma, may have arisen from the subcutaneous tissue, although it could also have been a mammary tumor. No other subcutaneous tumors were seen in any of these animals. The 4 bladders which were examined showed no lesions.

Subcutaneous injection of AAF solution.—It was previously mentioned (4) that 6 C57 mice, injected subcutaneously with AAF dissolved in sesame oil on 2 occasions, 42 days apart, appeared normal up to the time of death 10 months later. The seventh animal was autopsied 23 months after the first injection. There were no signs of tumors.

Five male rats were used for injections of AAF dissolved in propylene glycol. Injections of 20 mgm. of AAF in 0.4 ml. were made four times in a 6 month period. One animal which died after 238 days had myocarditis and renal lesions suggesting

* The experimental work on which this paper is based was completed in 1942, but because of the pressure of other work the authors were unable to prepare their results for publication.

[1]The AAF used for the experiments reported in this paper was from the same batch described in our first report (4).

chronic glomerulonephritis. There were no tumors. The rest lived for periods up to 614 days. One rat had leukemia. Another showed an infiltrating tumor of the lung composed of somewhat irregular spindle cells in a collagenous stroma. This has been classified as fibrosarcoma. It may have been the primary tumor, but there was a smaller subcutaneous nodule composed of similar tumor tissue. Another animal showed no lesions, and the fifth, from which only a section of bladder was studied microscopically had a small myoma of the bladder. Three bladders and four livers from these animals showed none of the changes usually seen after administration of effective doses of AAF so it is concluded that the compound given subcutaneously in propylene glycol in this quantity had little or no carcinogenic effect. The presence of leukemia in one animal which received propylene glycol alone and of a lung carcinoma and an adrenal adenoma in another animal similarly treated provides further doubt that the AAF administered in propylene glycol produced any lesions.

Installation of powdered AAF into the auditory canal.—We (4) have previously described subcutaneous epidermoid carcinomas of the side of the face and suggested that they may have arisen from the auditory canal or accessory structures such as sebaceous ducts. Bielschowsky (1) has definitely called the lesion a carcinoma arising from the ductus acousticus externus. While it was our belief that AAF had to be taken by mouth in order to be effective, it was possible that the animals got some of the compound into their ears while eating and that this might have caused the tumor in the head. To test this possibility, 6 male rats were used. Powdered AAF was blown into the ears weekly for 13 months. One animal died 21 months from the start and the others were killed and autopsied after 24 months. There was no gross suggestion of tumors in any tissue of any of the rats. Tissues from 5 of them were studied microscopically. They showed no liver or bladder lesions, or any of the other lesions common in AAF-treated animals. Two, however, had carcinoma of the lung. It seems likely that these were not specific results of the treatment, although some of the powder was visible in the air and a possible local effect from inhalation of the powder cannot be disregarded.

Effect of enriching the diet.—At about the time these experiments were in progress, it was being shown that the adequacy of the diet was of great importance in determining the carcinogenic effectiveness of *p*-dimethylaminoazobenzene. In particular, the addition of yeast to the diet decreased the incidence of hepatomas (*see* reference 2, p. 197). Since the liver tumors of the AAF animals frequently resembled certain of those produced by *p*-dimethylaminoazobenzene, it was decided to supplement an AAF diet with foods of a high vitamin content. To 79 parts of the regular diet were added 1 part of cod liver oil, 5 parts of dried brewers' yeast, and 15 parts of wheat germ. Sugiura and Rhoads (3) showed later that for a maximum effect, 15 per cent of yeast had to be incorporated into the rice diet containing the azo compound. However, since the regular diet used in the AAF studies seemed satisfactory from all viewpoints, as contrasted with the poor quality of the rice diet, it is probable that 5 per cent of yeast was an adequate addition.

Eleven rats, 6 females and 5 males, were placed on this enriched diet when they were about 45 days old. The concentration of AAF in the diet was 0.125 per cent for 41 days; it was then reduced to 0.031 per cent until the 143rd day, and afterwards omitted entirely. The vitamin supplements were discontinued after 215 days. By the 174th day of the experiment, 4 of the rats had developed external tumors and postmortem examination revealed possible thickening in the bladders and a somewhat nodular and cystic appearance of the livers. A fifth tumor was evident before the experiment was accidentally terminated. There was no indication that the dietary supplements had modified the types of lesions or changed the time at which they made their appearance. However, another study still in progress suggests that the diet may be of great importance in regard to the carcinogenicity of AAF.

Effect of the age of the rats.—Most of the animals used in these investigations were young, and were started on the experiment shortly after weaning. The relation of the age of the experimental animals to the carcinogenic effect of AAF was studied using 8 male and 6 female rats, aged 440 to 618 days. They were fed AAF in a concentration of 0.031 per cent in the diet for 274 days. In this length of time, 10 of the 14 animals had developed tumors and 3 of the others had organs, which although not normal, could not from gross appearance be considered tumorous. No histological examination was made. AAF was as effective in these older animals as in younger rats, but probably no more so. Because of the small number of animals, no conclusion is justified, but it is interesting to note that only 1 head tumor and 1 tumor of the mammary type were found in this group of 14 rats.

DISCUSSION

The earlier suggestion that AAF must be ingested for a considerable period is substantiated.

Installation of the powder into the ear was without effect, and although this was done but once a week, some of the compound presumably dissolved in the ear wax with resulting continuous exposure. The rats given AAF in propylene glycol subcutaneously developed a few tumors that were not typical of the lesions produced by AAF. The interpretation of these findings was complicated by the exhibition of several neoplasms in rats receiving only propylene glycol. Those rats in which subcutaneous implantations of AAF crystals were made developed lesions similar to those produced by oral administration for a barely effective period of time. The lesions were minimal, and a long time was required before they made an appearance.

It was suggested in the first paper (4) that the effective carcinogenic agent probably was not 2-acetaminofluorene, but 2-aminofluorene, deacetylation having taken place in the gastrointestinal tract. It is possible that the crystalline AAF was gradually hydrolyzed within the body producing the minimal lesions; or the continued presence of AAF in the body might have caused these changes. How long the AAF remained in the body is not known. The lumps at the site of implantation disappeared within a few weeks. Since AAF is soluble in fats and fat solvents, this disappearance was in all probability due to solution. Whether there was hydrolysis, or excretion or other metabolic change, it is not known. In any case, the development of tumors was not typical in that the incidence was low and the time required for development was long.

Apparently AAF must be taken by mouth for a period of time (at least 25 days) in order to produce cancer effectively. Introduction of AAF parenterally may lead to minimal changes after a prolonged time. It is possible that the effective carcinogen, even in this case, is aminofluorene, the acetyl group having been hydrolyzed off in the tissues instead of in the alimentary tract.

The carcinogenic action of AAF appeared at first to be much less subject to the nutritional status of the animal than is that of *p*-dimethylaminoazobenzene since the latter compound is most effective on a nutritionally unsatisfactory rice diet, and slight modifications of the diet greatly decrease the number of tumors developing. Addition of vitamin-rich foods to the already satisfactory diet did not change the incidence or time of development of AAF neoplasms. However, there is some recent evidence, still incomplete, that diet may be of considerable importance.

Spontaneous benign mammary tumors develop in about 3 per cent of the breeding female rats of the colony, especially in the older animals. This being the case, a more rapid or more severe development of cancer might be expected were older animals to be given AAF. However the effect on the older animals seemed comparable to that on young rats. The one possible difference, the apparent decrease in number of tumors of head and breast, is interesting, particularly in regard to the latter since these rats, some of which had been breeders, were at an age when spontaneous benign tumors might be expected to appear.

SUMMARY

1. A single, large dose of 2-acetaminofluorene (AAF) by mouth did not lead to the production of tumors. No carcinomas of the acoustic canal, and no lesions in other organs clearly attributable to AAF developed when powdered AAF was introduced into the external ear.

2. Subcutaneous injection of AAF dissolved in propylene glycol led to a few changes, some of them tumorous, which were not clearly the result of AAF. Implantation of crystalline AAF subcutaneously caused minimal changes after a prolonged time.

3. The most effective way found so far to produce cancerous lesions in rats from AAF is to administer the compound by mouth for a considerable time.

4. Enrichment of the diet by the incorporation of cod liver oil, yeast and wheat germ, did not change the incidence or time of development of AAF lesions. However, recent evidence indicates that the character of the diet is important.

5. Age of the experimental animal did not influence the time of development of AAF tumors.

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The Carcinogenic Activity of 2-Acetaminofluorene

IV. Action of Related Compounds

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Studies of the physiological action of 2-acetaminofluorene (AAF) were started because the compound had been found to have insecticidal properties, and it was desirable to have some knowledge of possible toxicity to mammals. When its carcinogenic properties were discovered (12), AAF was no longer considered for use as an insecticide. However, there were several other fluorene compounds that showed insecticidal promise, and therefore investigations were initiated to determine whether some compounds related to AAF were carcinogenic. Furthermore, such a study would probably aid in understanding the action of AAF.

EXPERIMENTAL

For the most part the compounds under investigation were incorporated in the diet and fed for some time to albino rats. This was the method which was found to be effective for AAF. The rats ate these diets for 100 days or more. The colony of rats has been described previously (12, 13).

2-Aminofluorene.—In a previous paper (12) it was suggested that the effective carcinogenic substance was 2-aminofluorene (AF) rather than AAF. This was because the introduction of AAF by parenteral routes (thus avoiding possible hydrolysis in the alimentary tract) did not seem to lead to tumors. The idea has been modified (13) since some of the animals that received AAF parenterally finally developed tumors, although nodular hyperplasia characteristic of the AAF effect either did not appear or was minimal and appeared late. It was noticed also in the original work that the rats excreted a substance which colored the pine shavings of the cage orange; solutions of AF of the proper concentration did the same, whereas solutions of AAF did not. This was probably a lignin color reaction with an amino compound (8). If so, then animals treated with AF should develop cancer.

Five female rats were given a diet containing 0.031 per cent of AF[1], and 6 were fed half this concentration or 0.016 per cent. The compound was administered for 403 days to the survivors. Those still alive 578 days from the start of the feeding were killed and autopsied. The 5 animals receiving the 0.031 per cent diet developed tumors similar in appearance and distribution to those seen in the rats fed AAF. The average time from the beginning of feeding to death was 410 days. Three of the 6 rats which received 0.016 per cent AF developed tumors, and the average time until death was 516 days. Postmortem examination revealed tissue changes similar to early AAF toxicity (cystic liver, enlarged rough liver, and small translucent spots in the lungs). Histological examination of tissue sections was made on 4 of the animals receiving 0.031 and on 2 of those on the 0.016 per cent diet. These 6 rats were the first of the group to die. The remainder did not differ from them materially in gross appearance, and tissue sections of these rats were not prepared. All the rats examined histologically showed distinct nodules of hyperplastic hepatic cells (Fig. 1) and various numbers of small cysts in the liver. In 3 of the 5 bladders examined histologically there were foci of nodular epithelial hyperplasia forming distinct papillomas (Fig. 2). Another bladder showed minimal epithelial irregularity while the remaining 1 showed no abnormality. Two animals

*The experimental work on which this paper is based was completed in 1942, but because of the pressure of other work the authors were unable to prepare their results for publication.

[1]This compound and the chlorofluorene, fluorene, fluorenone and xanthone were furnished by the Insecticide Division of the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

Melting points as reported in the literature for pure samples and as observed by us for the samples tested for carcinogenicity, are:

	Reported	Observed
2-Aminofluorene	129-130° C. corr.	126° C. corr.
2-Chlorofluorene	97-98° C. uncorr.	91° C. corr.
Fluorene	113-116° C. "	108° C. uncorr.
Fluorenone	84° C. "	81° C. "
Xanthone	173-174° C. "	162.5° C. corr.

Fortunately, the compound (AF) that proved to be carcinogenic was one of the purer compounds.

had squamous cell carcinomas of the head (Fig. 3), 2 showed nodular grouping of follicles in the thyroid, and there was one instance each of adenocarcinoma of the intestine (Fig. 4), bilateral adenoma of the adrenal medulla, focal epithelial hyperplasia of the renal pelvis, and mild irregularity of the pancreatic acini with pancreatic cysts. One lung showed irregular proliferation of mucous glands around a chronically inflamed bronchus and another showed two nodules of proliferated alveolar lining cells like those described in the AAF animals (3). The 2 ovaries which were examined had prominent clumps of large stromal cells; 1 contained several simple cysts, probably derived from follicles. One uterus showed extensive endometritis, while the other showed irregular grouping of small endometrial glands into indistinct nodules near the lining epithelium.

Concentrations of AF of from 0.016 to 0.25 per cent were fed to a series of C57 mice. On gross inspection, all 5 of the animals receiving the 0.25 per cent AF diet developed typical tumors. Histologically, 2 showed distinct adenoma formation in the liver, and 2 others had slight irregularity of hepatic cell arrangement in places, suggesting early nodular hyperplasia. Carcinoma of the bladder appeared in 3 and 1 showed slight thickening and metaplasia of the bladder epithelium. In 2 animals there was irregular thickening of the epithelium of the kidney pelvis, and 1 of these showed a squamous cell type of carcinoma in the kidney. A tiny nodule of tumor tissue in the lung of this animal was clearly a metastasis. No other hyperplasias or tumors were seen.

None of the mice on the lower concentrations had grossly recognizable tumors, although a number of the organs were not entirely normal. Two mice which were fed 0.125 per cent of AF in the diet showed irregular thickening of the bladder epithelium, and 1 had slightly thickened renal pelvic epithelium. Liver irregularity was slight in these animals and no distinct adenomas were seen. The mice on the lower dosages showed decreasing effects with decreasing concentrations. No distinct changes were found in any of the organs studied from the 5 animals which received 0.016 per cent of AF for 326 days.

This experiment was stopped earlier than the similar one with AAF (13), so that comparative effectiveness of the two compounds cannot be judged from a consideration of incidence of lesions. That AF is possibly more active than AAF in mice is suggested by what seems to be a slightly shorter time of incubation.

Crystalline AF (100 to 150 mgm.) was placed in a subcutaneous pouch of each of 5 rats, and 250 mgm. in the same way 96 days later. Considerable coloration of the shavings occurred for a few days following the implantations, then disappeared, indicating an initial rapid absorption and excretion. After the second implantation the wounds healed satisfactorily, but the animals refused food and water. Three of them died on the 112th day and were eaten so that autopsy was impossible. A fourth died on the 118th day. Gross inspection revealed no lesions; crystalline material was found at the site of implantation. Postmortem changes were considerable so that tissues were not saved for microscopic examination. The last animal was autopsied 477 days after the initial implantation. In this rat there were small liver nodules and cysts of the kind seen in animals fed AF, but the other organs were normal.

Five rats were given subcutaneous injections of AF dissolved in propylene glycol, 4 times in 6 months. Each injection contained 20 mgm. of AF. The experiment ran for 202 to 582 days. Two of the 4 animals studied histologically developed leukemia; leukemia developed in 1 of the 3 animals injected with propylene glycol alone. There were no distinct liver nodules or cysts although one animal showed slight irregularity in size of the liver cells. The 3 bladders examined were normal. One animal had a subcutaneous sarcoma and a bony proliferation of a leg suggesting osteoma; another had an adenoma of the adrenal medulla together with a nodule in the mesentary suggesting a chromaffin tumor. A third had a small papillary adenoma of the lung. These are not types of tumors which have been seen frequently in AF- or

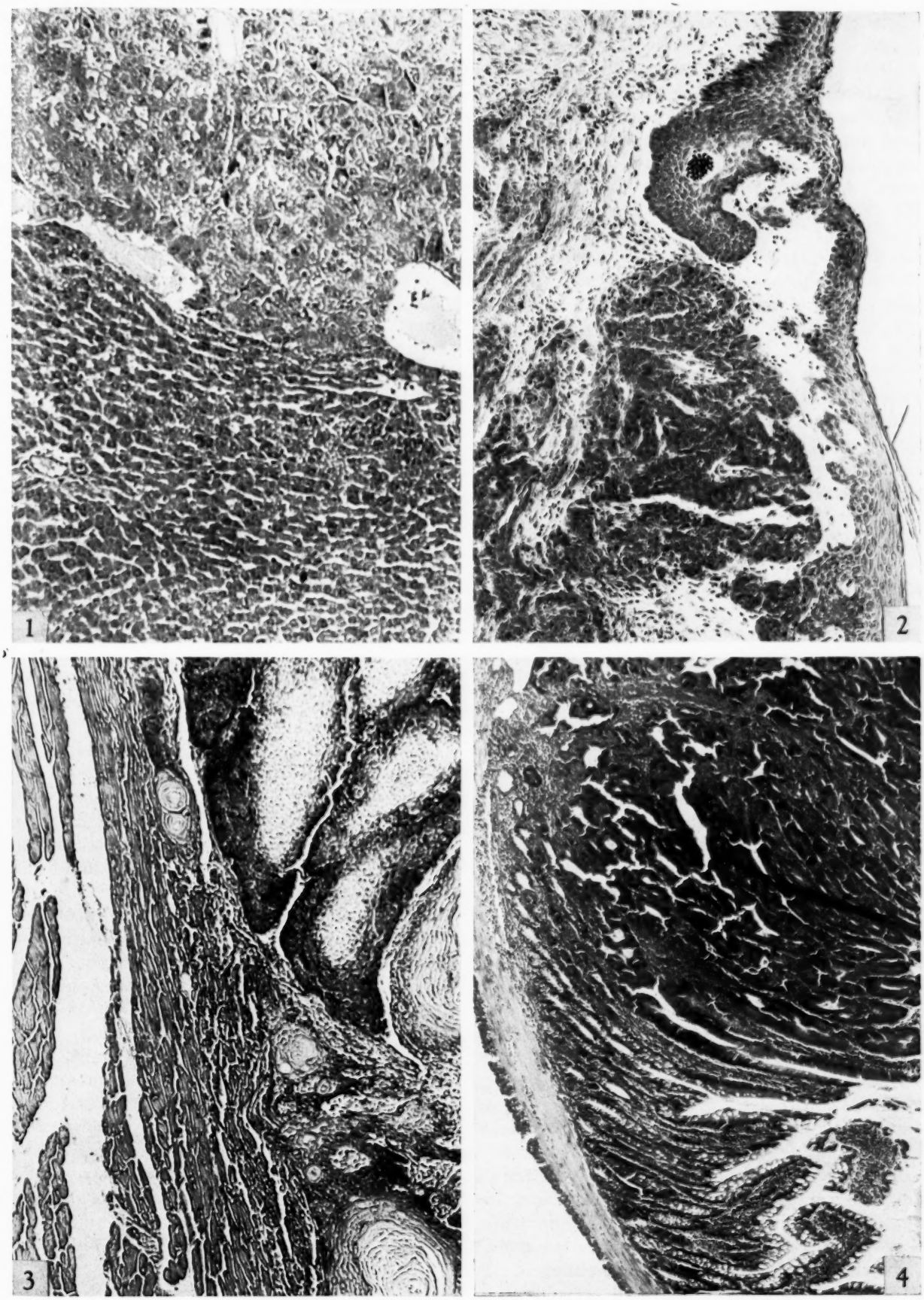
DESCRIPTION OF FIGURES 1 TO 4

FIG. 1.—Liver of Rat AF 1086. Margin of nodule of hyperplastic hepatic cells (above). Abnormal cells are larger and uniform. They do not penetrate among the preexisting liver cells, and there is no evidence of malignancy. Mag. × 100.

FIG. 2.—Bladder of Rat AF 1215. Carcinoma of bladder, showing infiltration of muscularis by groups of neoplastic epithelial cells. Mag. × 100.

FIG. 3.—Head of Rat AF 1109. Squamous cell carcinoma which arose adjacent to the external auditory canal, showing penetration of skeletal muscle by groups of tumor cells. The tumor is well differentiated and there is advanced keratinization in center of some of cell masses. Mag. × 100.

FIG. 4.—Intestine of Rat AF 1207. Hyperplastic nodule in mucosa of small intestine bordering a segment of normal mucosa. In this field there is little penetration of the muscularis, but in another place it is completely penetrated, indicating malignancy. Mag. × 100.



FIGS. 1-4

AAF-treated animals, so their significance is uncertain.

2-Chlorofluorene.—This compound differs from AF in having a chlorine in place of the amino group. Groups of female rats, five animals to a group, were fed chlorofluorene[1] in concentrations from 0.016 to 0.25 per cent of the diet. The highest concentration decreased the rate of growth to some extent. All rats except those on the diet containing 0.125 per cent were autopsied after 100 days. The organs were normal microscopically, and organ weights were normal with the exception of the heart, which averaged 13 per cent less than the average of control animals of the same body weight. This difference was statistically significant. The rats on the 0.125 per cent diet were continued until they had been on the diet for 337 to 602 days. Of the four animals autopsied, one had leukemia, and another had cysts of a mammary gland and an adenoma of the hypophysis. There was no other evidence of the type of abnormal cell growth seen in the animals fed AAF or AF.

Fluorene.—Fluorene has not been found to be carcinogenic when implanted subcutaneously or painted on the skin (for references, *see* [12]). However, it was considered advisable to administer it gastrically in the manner known to be effective for AAF. One series of rats was kept for 104 days on diets containing from 0.062 to 1.0 per cent fluorene.[1] A yellow staining of the fur around the urethral orifice was a common observation. Concentrations of 0.5 and 1.0 per cent led to decreases in the rate of growth which were statistically significant, but the general condition of the animals seemed good. Grossly and histologically the organs appeared normal, although weights of the organs were not. The livers of rats on diets of 0.25 per cent or more were significantly heavier than normal for animals of the same weight, the spleens of all groups of fluorene-treated animals were lighter than normal, and the testes of the rats on the highest dose were significantly small. Three groups receiving fluorene in the diet in concentrations of 0.125, 0.25 and 0.5 per cent were observed for 453 days. At autopsy they were fat, healthy and showed no gross signs of tissue damage. The yellow fur was the only indication that these animals were not on a normal diet. Histologically, several animals showed pulmonary inflammation which was unrelated to the dose of fluorene. In three of these there was metaplasia of bronchial epithelium to squamous type. One animal on the 0.125 per cent diet showed a small benign tubular adenoma of the kidney of a type not seen before in this colony. There was no other metaplasia or hyperplasia of the types seen in animals fed AAF. Four of these animals had moderate testicular atrophy, 2 had pericarditis, and two showed bladder worms.

Five milligrams of fluorene dissolved in propylene glycol was injected subcutaneously in each of 4 rats 3 times in a 6 month period. Tissues from 2 of these were examined histologically. One of them showed leukemia as did 1 of the rats receiving propylene glycol alone.

Fluorenone.—Fluorenone[1] is the compound obtained by replacing the two hydrogens of the 9-carbon of fluorene with an atom of oxygen. It was incorporated into the diet in concentrations of 0.031 to 0.5 per cent. Concentrations of 0.125 per cent or more of fluorenone significantly decreased the rate of growth, especially in the latter half of the 100 day feeding period. The livers, kidneys, and perhaps testes of these rats were significantly heavier than in the appropriate controls. The fur was stained yellow or yellow-brown. There were no indications of tumor or of nodular hyperplasia. Six female rats were placed on a 0.25 per cent fluorenone diet for periods ranging up to 600 days. Among these, 2 of the animals had small thyroid follicles and 1 showed a cystic endometrium.

Twenty milligrams of fluorenone dissolved in propylene glycol was injected subcutaneously into each of 4 female rats 3 times during a 6-month period. The animals were observed for 373 to 600 days. Two of the animals had breast adenomas—in 1 there was nodular hyperplasia of additional mammary tissue. One animal had leukemia. There were no other hyperplastic lesions like those in the AAF animals.

Diethylaminoethyl-fluorene-9-carboxylate hydrochloride.[2]—The compound is a fluorene derivative with substitution in the 9-position. It has been shown to have certain atropine-like actions (7). This compound was mixed with the diet in a concentration of 0.031 per cent and fed to 6 female rats for 431 days. The growth rate of these animals did not differ significantly from that of control rats. Several of the rats had pneumonia when autopsied, but none had anything suggesting tumors.

Xanthone.[1]—This compound is another which was of interest to the Bureau of Entomology and Plant Quarantine because of insecticidal action. It differs from fluorenone in that an oxygen in an ether linkage is inserted between the 2 benzene rings. Xanthone affected the rats in a way similar to fluorene. There was occasional yellow or orange

[2]The diethylaminoethyl-fluorene-9-carboxylate hydrochloride was furnished to us by Dr. P. J. Hanzlik, Dept. of Pharmacology, Stanford University School of Medicine, who received it through the courtesy of G. D. Searle & Co. We have no knowledge of the purity of this compound.

coloration of the fur. Animals on the diet containing 0.5 per cent of the compound had a significantly slower rate of growth than did control rats, and growth on the 1.0 per cent diet was practically stopped. After 100 days on the diet, rats eating 0.25 per cent or more of the compound in the diet had livers heavier than normal for their body weights, and their spleens were slightly lighter. The organs appeared normal. Five female rats were kept on the 0.25 per cent diet for over a year, and 3 of them for 640 days. The organs of all appeared normal at autopsy.

DISCUSSION

As had been anticipated, AF proved to be carcinogenic. There are, however, certain apparent discrepancies. If AAF is active through the AF formed from it by hydrolysis in the intestinal tract, then AF should be at least as active as AAF. This was not the case with the rats. Lesions were slower in developing, and at the 0.016 per cent level, were less numerous. This may be due to differences in solubility. AF is considerably more soluble than AAF. Perhaps in the rat it was absorbed soon after ingestion and excreted quickly thereafter, so that the tissues were not continually exposed to the compound. The AAF, on the other hand, may have given a more continuous exposure, because AF could be absorbed only as fast as the acetylated compound was hydrolyzed in the intestine. In mice, on the other hand, the AF seemed to be as effective as AAF, and perhaps a little more so, as judged by the time of development of tumors. Possibly a difference in eating habits could explain the divergent findings of rats and of mice.

The comparatively slight effect noted in the 1 rat that survived 2 subcutaneous implantations of crystalline AF may also be explained by an influence of solubility and rate of excretion upon carcinogenic effect. That the AF was absorbed to a considerable extent is clear from the fact that after the second implantation, all 5 of the rats were sick and 4 of them died. Similar implantation of AAF crystals in other rats had no observable effect.

Bielschowsky (1) has described an experiment in which he obtained distant malignant tumors in rats that had had their skins painted with AF. A 4 per cent solution in acetone was applied thrice weekly for 210 days. This experiment differed in certain important respects from the 1 described in this paper where crystals of AF were implanted subcutaneously. The frequent painting and the presumably slow absorption from the skin might keep a continuous stream of the compound passing through the animal. That AF is carcinogenic is clear from the work of Bielschowsky and the data presented in this paper.

If 2-chlorofluorene is changed at all to AF by the animal, the extent of the change is probably very slight. The hydrocarbon fluorene, the 2 fluorene compounds in which substitution was at the 9-carbon, and xanthone, which differs from fluorenone by having an oxygen atom introduced into the central ring, all proved to have no carcinogenic action when given to the animals together with the food. As mentioned earlier, others have found that fluorene is not carcinogenic when applied to the skin or implanted underneath it.

In an earlier paper (12) we suggested that an amino group in the 2-position might be of importance in determining carcinogenicity of certain compounds—2-aminofluorene, 2-acetaminofluorene, beta-naphthylamine (5), 2-aminoanthracene (10), 2, 2′-diamino-1, 1′-dinaphthyl, etc. (2). The present paper does not give any final answer to this suggestion, but it does show that aminofluorene is carcinogenic, that replacement of the amino group by chlorine destroys that activity, and that certain substitutions at positions other than the 2-carbon give non-active compounds.

A number of the compounds were given subcutaneously in propylene glycol solution. In several instances, and apparently without regard to the amount or character of the dissolved compound, death of the animal from leukemia occured. Indeed leukemia developed in 1 of 3 rats given subcutaneous injection of propylene glycol alone. Altogether there were 21 control and experimental rats that received propylene glycol subcutaneously; 6 of these developed leukemia in 202 to 600 days. While occasional instances of leukemia have been noted in AAF animals, the incidence was much lower than for the rats receiving propylene glycol solutions of the various substances; in non-experimental rats and in rats used for other studies, leukemia has not been observed. Propylene glycol has been studied somewhat extensively and has not been considered a very toxic substance. Dogs have been kept for 5 to 9 months on a regimen in which their fluid requirements were met by a 5 per cent solution of propylene glycol (11). Hanzlik, Lehman, Van Winkle and Kennedy (4) fed rats for 5½ months on a diet that was 25 per cent propylene glycol. Kesten, Mulinos and Pomerantz (6) administered the glycol in drinking water to rats for times up to 234 days. It is possible that in these instances the animals were not observed for a long enough period to permit the development of leukemia. However, this cannot be said

of the study by Morris, Nelson and Calvery (9), who kept rats on diets containing 2.45 and 4.9 per cent of the compound for 24 months. The high incidence of leukemia in the propylene glycol-injected animals of this colony deserves further study.

SUMMARY

1. 2-Aminofluorene when incorporated in the diet and fed for a considerable period to rats and mice, produced carcinomas resembling those formed by 2-acetaminofluorene. Implanted crystals of aminofluorene were quite toxic; in the rat that lived, lesions were slight. This may be due to the elimination of the compound from the body before malignant processes were well initiated.

2. 2-Chlorofluorene, fluorene, fluorenone, diethylaminoethylfluorene-9-carboxylate and xanthone were nor carcinogenic when administered in the diet to rats.

3. It is suggested that an amino group in the 2-position is important in determining carcinogenic activity of certain types of compounds.

4. Leukemia developed in a number of rats given propylene glycol solutions subcutaneously. The literature does not record any leukemias in animals given the compound by mouth. It should be determined more definitely whether the glycol was the causative factor.

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The Cytotoxic Effect of Avian Lymphoid Tumor Antiserum

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A cellular suspension prepared from a lymphoid tumor (11), when injected into the pectoral muscle of chickens, will produce a rapidly growing tumor at the injection site which will metastasize to the viscera in a high percentage of cases. Burmester, Prickett, and Belding (3) demonstrated the presence of a filtrable agent which, though incapable of producing a tumor at the site of inoculation, caused the occurrence of a high incidence of osteopetrosis and lymphoid tumors of the viscera during an experimental period of 6 months.

It was found (2) that all birds which survived takes of the first cellular implant of this tumor were invariably highly immune to a second or a third implant and that this immunity persisted for at least 202 days. Olson (12-14) treated tumor suspensions with heat, cold, chemicals, drying and centrifugation and found generally that when the growth capacity was destroyed the immunizing ability was likewise lost.

This report describes the demonstration of an antibody-like factor in the serum of lymphoid tumor immune birds and in other birds which have received repeated injections of tumor material without viable cells. The factor was identified by its *in vitro* toxic effect upon tumor cells and by its *in vivo* effect when injected into birds before and after receiving an implant of tumor cells.

I. CYTOTOXIC ACTION *IN VITRO*

Materials and Methods

The general procedure used in testing the *in vitro* effect of antiserum was to incubate the lymphoid tumor cells in the serum to be tested, after which the mixture was injected into the pectoral muscle of 5 to 11 day-old chicks. Formation of a palpable tumor within 3 weeks at the site of inoculation was used as the measure of the viability of the tumor cells. This criterion was based on the finding of Burmester, Prickett, and Belding (3) that inocula containing tumor cells produced palpable tumors at the site of injection in 7 to 14 days; whereas cell-free extracts failed to produce tumors at the site of injection. However, chicks inoculated with such extracts developed tumors of the bone and viscera in 90 to 180 days. The avian lymphoid tumor used in this investigation was developed by Olson (11) and known at this Laboratory as strain RPL 12.

Chickens used for the propagation of the tumor, the source of serum, and the source of baby chicks for the cytotoxic tests in all experiments except numbers 10 to 15 inclusive, was an inbred line of chickens selected for its high susceptibility to naturally occuring lymphomatosis (16). Matings that supplied the chicks were maintained in strict isolation and had a low incidence of lymphomatosis (15). Chicks for Experiments 10 to 15 were the progeny of the crosses of two inbred lines developed at this Laboratory (16). All birds used in hyperimmunization were 200 or more days of age.

Lymphoid tumor antiserum.—Antiserum and plasma prepared by the use of a fresh suspension of tumor cells were made in 3 different series of injections involving different groups of chickens. Series I consisted of a group of chickens that survived the original tumor cell implant and were subsequently found refactory to a second implant made 21 to 39 days later. After an interval of 148 to 220 days, the birds were given ten 1 cc. portions of fresh tumor mince injected intramuscularly at weekly intervals. Immune plasma from this series was collected 6 days after the seventh hyperimmunizing injection. The inoculum used for the repeated injections was prepared by pooling several tumors of the pectoral muscle of birds used in the serial passage of strain RPL 12. The tumor tissue was minced (10) and suspended in equal parts of Tyrode's solution.

Series II chickens were hyperimmunized a year later by essentially the same procedure used in Series I except that fewer injections of tumor mince were made prior to the collection of blood. The plasma used for testing the cytotoxic effect in the blood of birds of Series I and II was obtained by drawing blood from the heart into a syringe containing 0.1 volume heparin solution having a concentration of 0.4 gm. per 100 ml. of 0.85 per cent NaCl solution. The blood cells were sedimented by centrifugation, the plasma decanted and stored at about 2° C. until used in the tests.

In the hyperimmunization of birds in Series III, the following 5 different preparations were used:

(a) *Tumor cell suspension, unfrozen.*—Antigen "a" was prepared by mincing a composite of several intramuscular tumors from birds in the serial passage of this tumor strain. The mince was diluted with 3 parts of saline and injected into birds that had survived a previous implant of viable tumor cells.

(b) *Tumor cell suspension, frozen rapidly and thawed.*—Antigen "b" was prepared in the same manner as "a," except that, in addition, it was sealed in pyrex tubes and immersed in ethyl alcohol cooled to −76 C. by solid CO_2. After 5 minutes the inoculum was thawed in running tap water. This freezing and thawing procedure was repeated twice.

(c) *Tumor cell suspension, heated.*—Antigen "c" was prepared the same as "a" except that in addition the suspension was heated to 50° C. and held at that temperature for 30 minutes in a constant temperature water bath.

(d) *Homogenized tumor suspension.*—Tumor material for this preparation, antigen "d," was obtained from the same source as for the "a" antigen. It was ground in a Waring Blendor[1] for 10 minutes with 3 parts of normal saline. The temperature of the material was maintained in the range of 2° to 10° C. by cooling at intervals in ice water mixture during the process.

(e) *Ground normal tissue suspension.*—The bursa of Fabricius, thymus, spleen, and a part of the liver from several birds raised in isolation, and which showed no gross evidence of disease, were pooled and processed in the same manner as "d." This antigen, "e," served as the normal tissue control.

Antigen "a" was injected into birds that had survived a previous implant of the same tumor strain. Preparations "b," "c," "d," and "e," were injected into male birds not previously inoculated. Four to 6 birds were used for each preparation. Injections were made every other day for 24 days. The first 2 injections consisted of 2 cc. of the antigen given intramuscularly and the remaining 10 injections of 4 cc. were given by the intraperitoneal route. No tumors developed in any of the birds injected.

Blood was collected on the sixth and 12th days after the last hyperimmunizing injection was made. The blood was allowed to clot and the serum separated with the aid of a centrifuge. The serum was sealed in glass serum tubes and stored a maximum of 213 days at 2° C. until used.

[1]Obtained from Central Scientific Co., Chicago, Illinois.

Normal serum.—Serum was also collected from birds raised in isolation and without previous or concurrent evidence of disease. All such serum from "D" and "G" ("D" and "G" designate the chickens that were hatched during the calendar years 1942 and 1945 respectively) birds was frozen and stored at −76° C. whereas all other serum was stored at 2° C. until used.

Lymphoid tumor cells.—Cells used for testing the cytotoxic activity of the antiserum were obtained from intramuscular tumors of birds in the serial passage of strain RPL 12. The excised tumor was minced and suspended in 2 parts of saline and then filtered through 2 layers of cheese cloth. The concentration of tumor cells was estimated by the use of a standard blood cell counting chamber and appropriate dilution made to obtain the desired cell concentration before the addition of the antiserum to be tested. All tests were made by adding 9 parts by volume of antiserum to 1 part of tumor cell suspension. The total volumes used varied from 0.2 ml. to 1.0 ml. The mixture was then incubated for 2 to 24 hours at temperatures of 2° to 37° C. The actual factors used depended upon the experiment. For most experiments antiserum from individual birds was used, but in certain instances (Table II) antisera or plasma from several birds of the same treatment were pooled. All results reported in Tables II and III were obtained with mixtures incubated for 24 hours at 37° C. Immediately following the incubation period, 0.02 to 0.05 ml. of the mixture was injected into the right pectoral muscle of chicks of 5 to 11 days of age. The number of tumor cells per injection dose was about 1,000 for all experiments except 8 and 9, 200 cells being used for the former and 2,000 for the latter experiment. Chicks were examined for the presence of a tumor every 3 days during the period beginning with the 10th day and ending with the 19th day after inoculation. New tumors appeared very infrequently after the 16th day and only a few tumors grew after the 19th day. Since all results reported are based on the presence or absence of palpable tumors, the maximum incidence (± experimental error) was no doubt attained by the 19th day. An additional 2 days were allowed and all birds were killed and examined on the 21st day after inoculation. No lesions other than tumors were found in the inoculated chicks.

Results of inoculation are presented in the tables in terms of the fraction, that is, the number that developed tumors over the number that were inoculated. Only those with a distinct tumor growth were classified as positive. The few questionable cases that did occur were classified as negative. The activity of the tumor cell suspen-

sion and the effect of normal serum were tested in each experiment to provide a basis for comparison with the tumor cells treated with the antiserum.

RESULTS AND DISCUSSION

The toxic effect of hyperimmune plasma on lymphoid tumor cells was detected in the first experiment, in which the cells were suspended in immune plasma for 2 hours and for 20 hours prior to their injection into young chicks. The mixture that was incubated for 2 hours produced tumors in 5 of 10 chicks injected and that incubated for 20 hours produced tumors in only 2 chicks of 9 inoculated. Tumor cells from the same source when incubated in normal plasma for 20 hours produced tumors in all of 9 birds injected.

TABLE I: INFLUENCE OF TIME AND TEMPERATURE ON THE CYTOTOXIC ACTION OF ANTISERUM AND PLASMA

Exper. No.	Incubation		Tumor incidence*	
	Time (hrs.)	Temperature (degrees C.)	Lymphoid tumor antiserum	Normal Serum
1†	2	7	5/10	9/10
	20	7	2/9	9/9
2†	2	2	9/10	
	2	18	10/10	
	2	37	11/11	
	24	2	7/11	11/11
	24	18	0/10	10/10
	24	37	0/10	9/9
15	3	2	6/7	
	3	20	2/7	
	3	37	3/7	
	8	2	5/7	
	8	20	4/7	
	8	37	2/7	
	24	2	3/7	7/7
	24	20	1/7	7/7
	24	37	0/7	7/7

* Number that developed tumors/number inoculated.
† Plasma used.

These results suggested that plasma of birds that had survived implants of this lymphoid tumor and had received several hyperimmunizing injections, materially decreased the ability of tumor cells of the same strain to reproduce and grow into a detectable tumor in the new host. There was also some indication that the length of the incubation period of the mixture may have influenced the results.

Influence of time and temperature.—The influence of the length of the incubation period and the incubation temperature are shown in the results of 3 experiments presented in Table I. Results of the first experiment, as indicated above, showed that with an increase in the length of the incubation period from 2 to 20 hours, the tumor incidence was reduced from 5/10 to 2/9. The difference, however, is not statistically significant.

In the second experiment, incubation temperatures of 2°, 18° and 37° C. were used for periods of 2 and 24 hours. No toxic effect was obtained when the mixture was allowed to stand for only 2 hours. However, mixtures that were held for 24 hours showed a marked reduction in their tumor-producing activity. Tumor cell-immune plasma mixtures held at 2° C. for 24 hours, produced tumors in 7 of 11 chicks inoculated, but similar mixtures held at 18° and 37° C. for the same length of time produced no tumors. Tumor cells suspended in plasma from normal birds and incubated at the latter temperatures and time produced tumors in all chicks injected.

In a third experiment (No 15), tumor cells were suspended in hyperimmune serum for 3, 8, and 24 hours each at 2°, 20°, and 37° C. The results (Table I) showed that at each time period the toxic effect increased with the temperature, with the exception of the 3 hour, 20° group. This group had fewer birds with tumors than did the 3 hour, 37° group. The results also showed that within each temperature group the toxic effect increased (again with the exception of the 3 hour, 20° group) with an increase in the length of the incubation period. Thus, the cytotoxic effect increased with an increase in incubation time and temperature so that the maximum effect (no tumors in 7 birds injected) was obtained in the 24 hour, 37° C. group. This was the maximum for these factors in experiments thus far conducted. No such effect was obtained when the tumor cells were incubated in normal serum. All birds that received tumor cells incubated in normal serum for 24 hours developed tumors.

Other investigators working with other materials have used a much shorter incubation period to demonstrate a cytotoxic effect of antiserum. Kidd (8) obtained a suppression of tumor growth when the transplanted Brown-Pearce tumor cells were first incubated with antiserum for only 2 to 3 hours at 37° C. Green (7) incubated mammary cancer cells in mammary cancer milk agent antiserum for 3 hours at room temperature and then 3 hours at 7° C. and obtained a complete suppression of tumor growth.

The reason for the much longer reaction period necessary for maximal effect with the avian lymphoid tumors is not evident. Some cytotoxic effect was obtained in experiment 15 when the incubation time was only 3 hours, 2 of 7 birds developed tumors when the incubation temperature was 20° C. and the incidence was 3 of 7 when the temperature was 37° C. thus indicating that a measureable toxic effect occurs within 3 hours. That the differences may be due to a low concentration of antibodies in the serum should be

considered. Preliminary titrations indicate that the cytotoxic activity of sera thus far tested was quite low. In the first such trial (not presented in Tables) tumors grew in only 3 of 10 birds injected with tumor cells incubated in undiluted serum; whereas cells suspended in antiserum diluted 1:5 and 1:25 with normal serum, produced tumors in 8/10 and 9/10 birds, respectively. In a second test, serums from 5 different hyperimmunized birds were used. The results for the different sera were quite consistent, giving a total of 3 birds with tumors of 20 inoculated with cells suspended in the undiluted serum, and 17 with tumors of 20 implanted with cells suspended in serum diluted 1:5 with normal serum. Tumor cells suspended in normal serum again produced tumors in all birds injected. Although the incidence of tumors produced by cells suspended in diluted antiserum was high, the maximum size of the tumors and the mortality were much lower than in the normal serum group. Thus no suppression of growth was obtained with antiserum diluted 1:5. However, the effect was much less than with the undiluted antiserum. Since undiluted serum was necessary for maximal effect, then it may be inferred that a long reaction time must be used to obtain the maximal effect.

Influence of source of antiserum.—While conducting numerous tests under similar conditions but with various antisera, it was found that the apparent cytotoxic activity varied. Results of various tests in which the cells and sera were incubated for 24 hours at 20° to 37° C. are presented in Table II. Results with lymphoid tumor antisera are from 9 different tests, using antisera from 3 different (see methods) hyperimmunizing series. Five individual samples of the antisera and 5 composite samples made by pooling the serum of several hyperimmunized birds were used. No bird contributed to more than one pooled sample.

TABLE II: INFLUENCE OF SOURCE OF SERUM ON THE CYTOTOXIC PROPERTIES OF NORMAL SERUM AND ANTISERUM

Exper. No.	Series	Lymphoid tumor antiserum		Normal tissue antiserum		Normal serum	
		Source	Tumor incidence*	Source	Tumor incidence*	Source	Tumor incidence*
1	I	Pooled†	2/9			Pooled†	9/9
2	II	Pooled (a) †	0/20			G1319A2†	19/19
3	II	Pooled (b) †	7/7			D821A2	7/7
8	III	Pooled (a)	0/10			G1319U2	7/10
9	III	G1202H2	0/5	G1202T2	5/5	G1319U2	5/5
11	III	Pooled (b)	3/5		4/4	G1319U2	4/4
12	III	G1202H2	1/4	G1202T2	3/4	G1319U2	1/4
		G1255J2	1/4	G1210R	4/4	D821A2	4/4
		G1270J3	1/4			D805Q2	4/4
		G1319P3	0/4				
		G1324Z2	0/4				
13	III	G1202H2	0/12	G1202T2	5/6	D821A2	6/6
		G1319P3	1/12	G1210R	6/6	D821S	6/6
		G1324Z2	4/12			D805G	6/6
						H1310N	6/6
						H1310J	6/6
						H1313G	6/6
						H1313K	6/6
14	III	G1202H2	0/5	G1202T2	3/5	D821S2	5/5
		G1255J2	0/5	G1210R	4/5	D821K2	3/5
		G1270J3	0/5			D821Z2	5/5
Total			20/127		34/39		115/123
Percentage			15.7		87.2		93.5

* Number that developed tumors/number inoculated.
† Plasma used.
(a) (b) See description under Methods.

Results obtained vary from a low incidence of tumors of 0/20 for composite "a" of hyperimmunizing series II, to a high of 7/7 for composite "b" of series II. A low incidence of no tumors occurred in most of the tests conducted. In almost one-half of the tests (9 of 17) none of the birds developed any gross evidence of tumors. Of a total of 127 birds used in testing the cytotoxic activity of the 17 different sera, only 20 birds (15.7 per cent) developed tumors and over one-third (7) were from the one high incidence test cited above (composite "b," series II).

Sera of 2 birds (G1202T2, G1210R in Table II) that had received repeated injections of minced normal lymphoid tissue were tested for their cytotoxic effect with the same lymphoid tumor cell suspensions used with the lymphoid tumor antiserum. In the 7 different tests made, the incidence

of tumors varied from 3/5 to 6/6, giving an average of 87.2 per cent for the 39 birds used in the 7 tests.

Lymphoid tumor cells used in testing the cytotoxic activity of the various antisera were in each test also suspended in serum from normal birds, prior to their implantation, in order to test the growth activity of the tumor cells and the effect of the incubation period and temperature. The sera from 14 different normal birds were used in 19 different tests. A 100 per cent incidence of tumors was obtained in all except 3 tests and 2 of these were with the same serum samples (G1319U2). However, a 100 per cent incidence was obtained in 2 other tests, using the same serum. Thus, a considerable variation in results was obtained in several tests of the same normal serum. This error in the determination is no doubt due in part to the unrecognized variation in the test animals, material and technics. It should also be recognized that birds that were used as the source of normal serum or antiserum may have carried a low titer of similar antibody-like factors, *i. e.*, thay may have had lymphomatosis in an early or inapparent form.

Duran-Reynals (5) found that antibody-like factors endowed with the property of suppressing the effect of viruses of Rous and Fuginami sarcoma develop in the blood of normal fowls paralleling the growth of the individual. No indication of a phenomenon of this sort was found in working with materials and birds reported herein. Untreated birds having band numbers with the prefix "D" supplied the normal serum when they were 300 days of age. Birds with the prefix "H" supplied the normal serum when 60 to 110 days of age. In a test, the results of which are not given in Table II, serum from untreated birds 16 days of age was used. Results with serum of birds of all age groups were similar in that no cytotoxic effect was obtained, indicating that the antibody-like factor of Duran-Reynals, which is present in normal adult fowls, does not appear to play a part in the suspension of the growth of lymphoid tumors herein described.

Results presented in Table II suggest that there is some variation in the cytotoxic activity of various lymphoid tumor antisera. The variation in results obtained in several determinations of the same serum, indicate that some of these variations may be due to errors inherent in the method.

In testing Tyrode's solution as a possible diluent for tumor cells and antisera, it was found that when the tumor cells were suspended in Tyrode's solution for only 2 hours at 20° C., some growth activity resulted. When the holding period was increased to 24 hours the growth activity was completely lost in two such tests; however, when as little as 10 per cent by volume of normal serum was added to the Tyrode's solution, the tumor cells retained their full tumor inducing activity after 24 hours at 20° C.

Influence of heating the antiserum.—Sera from 2 hyperimmunized birds were heated to 56°, 61° and 66° C. for 30 minutes by placing 2 cc. portions of the serum in small test tubes and then immersing the lower half of each tube in a constant temperature water bath, the fluctuation of which was not more than 0.5° C. After cooling again to room temperature, lymphoid tumor cells were added to the various tubes of heated and unheated antiserum and to a tube of unheated normal control serum. After each mixture was incubated at 37° C. for 24 hours, it was injected into 7 chicks. The results obtained gave no indication of a decrease in cytotoxic activity even at the highest temperature used (66° C.). No tumors developed in any of the birds that received cells suspended in heated or unheated antiserum, whereas all of 7 birds inoculated with tumor cells suspended in normal serum developed tumors.

Influence of using killed or disintegrated tumor cells on the activity of the antiserum.—Chickens injected with antigens "b," "c," and "d," as described in the section on methods and materials, had received no previous injection of cellular or cell-free lymphoid tumor material and they did not develop tumors after injection of the antigens. It can, therefore, be assumed that antigens "b," "c," and "d" were relatively free of viable tumor cells, since it is known that relatively few viable tumor cells are necessary for the initiation and growth of this tumor in chickens.

The antigens containing killed or disrupted tumor cells produced no visible reaction in the birds receiving the several injections during the hyperimmunization period. However, results of 4 experiments testing the cytotoxic activity of antisera produced with killed cells, indicated that an antibody-like factor was present in the sera, similar to that found in birds which had received viable tumor cells.

Results of these experiments are presented in Table III. Tumor cells incubated in antiserum against rapidly frozen and thawed cells, produced no tumors in 4 tests and did produce tumors in ⅕ to ½ of the birds in 3 other tests, making a total of 6 positive out of 35 injected. Tumor cells incubated with antiserum against heated cells, produced no tumors in 2 tests. Tumor cells suspended in antiserum against tumor material which had been homogenized in a Waring Blendor, caused no tumors in 6 tests with 28 chicks. That all cell sus-

pensions used in testing the sera were highly active in all tests is indicated by the 100 per cent incidence obtained when the suspensions were incubated with normal serum. These results indicate that the cytotoxic activity of the serum was not dependent upon a viable and functional cell.

TABLE III: CYTOTOXIC ACTIVITY OF ANTISERUM PREPARED WITH RPL 12 TUMOR CELLS KILLED BY RAPID FREEZING AND THAWING, BY HEAT TREATING, AND BY MECHANICAL DESTRUCTION

Tumor incidence*

	Lymphoid tumor antiserum made with antigen			
Exper. No.	(b) Frozen and thawed 3 times	(c) Heated to 50° C. 30 min.	(d) Homogenized 10 min.	Normal serum control
9	1/5		0/5	5/5
12	0/4	0/4	0/4	4/4
	0/4		0/4	
13	3/6	0/6	0/4	6/6
	2/6		0/6	
14	0/5		0/5	5/5
	0/5			
Totals	6/35	0/10	0/28	20/20
Percentage	17.1	0	0	100

* Number that developed tumors/number inoculated.

The foregoing experiments show conclusively that an antibody-like factor may be produced in the serum of birds which inhibits partially or completely the growth of the cells of this avian lymphoid tumor. This effect was obtained with serum of birds surviving several implants of viable tumor cells, as well as with serum from birds receiving several injections of tumor cells killed by freezing and thawing, by heat, and by homogenization. Thus, it would appear that the antigenic unit was a constituent of the tumor cell.

The failure to produce a similar antibody by the repeated injection of lymphoid tissue from normal birds prepared in a manner similar to the lymphoid tumor preparation would indicate that isoantigens were not involved. Since the antigenic material came from the same inbred line as the hyperimmunized birds antigenic differences between individuals were presumably much less than if unrelated individuals had been used.

The cytotoxic effects obtained with this avian lymphoid tumor are similar to the results reported for 2 mammalian tumors. Green (7) prevented completely the growth of transplanted mouse mammary cancer by mixing the tumor cells with its antiserum 6 hours before implantation. Normal mammary antiserum and normal rabbit serum produced only a slight inhibition of growth. A similar antiserum was found to neutralize the mouse mammary-tumor agent (1, 6). A suppression of growth of Brown-Pearce tumor cells by a specific antibody *in vitro* and *in vivo* was demonstrated by Kidd (8, 9). Such antibodies were found in the serum of rabbits in which an implanted tumor had regressed and in some rabbits that had received several injections of saline extracts of the tumor. Antiserum which was heated to inactivate the complement retained its antiproliferative effect.

Although it is known that extracts of lymphoid tumor will induce tumors in the viscera (3), experiments have thus far not been conducted to test the relationship between this filtrable tumor-inducing agent and the antigenic agent described herein.

II. *IN VIVO* EXPERIMENTS

PROCEDURE AND RESULTS

General.—The procedure employed for testing the *in vivo* effect of hyperimmune plasma was to make repeated injections into young chicks that had received implants of lymphoid tumor cells.

The chicks used in Experiments 1 and 2, and for one group of Experiment 3 (Stock A) were from the same lymphomatosis-susceptible, yet relatively disease-free, line of chickens which supplied chicks for all *in vitro* experiments with the exception of numbers 10 to 15. Stock B chicks used for 2 other groups of Experiment 3 were obtained from several matings of birds from lines susceptible to lymphomatosis (16). The birds used in these matings were classified as contaminated stock because a relatively high percentage of lymphomatosis occurred naturally in their progeny. The hyperimmune plasma used for the injections was collected from the series I group described in the previous section.

The presence of a tumor at the site of inoculation was determined by palpation at 3 day intervals and by examination at autopsy if the bird died.

Experiment 1.—Thirty-one chicks 7 days of age received 200 lymphoid tumor cells (RPL 12) by injection into the deep pectoral muscle. Six of these birds did not receive plasma injections. The remaining 25 chicks were divided into 5 groups and received daily injections of 2 ml. of hyperimmune plasma. The route and number of injections before and after implantation of the tumor cells varied with each group. Five birds that did not receive implants of tumor cells were also given the maximum number of plasma injections. The treatment of each group and the results obtained are presented in Table IV. The longest plasma injection period was 2 days before to 10 days after implantation inclusive: the shortest, 8 days after to 10 days after implantation inclusive. The incidence of tumors among the five groups during

the 28 day post-tumor implantation period varied from 5/5 to 3/5 and the survival from 3/5 to 4/5. Neither measure showed any particular relation to the number of hyperimmune plasma injections given. The 6 birds that received no plasma died with tumors and the 5 birds that were not injected with tumor cells, but received the maximum number of plasma injections, survived and showed no evidence of tumors.

TABLE IV: EFFECT OF INJECTING HYPERIMMUNE PLASMA INTO CHICKS IMPLANTED WITH LYMPHOID TUMOR STRAIN RPL 12

	Plasma injections before(−) and after (+) tumor cells implanted	Number injected	Number with tumors	Number surviving
Experiment 1 (*Stock A*):				
Hyperimmune plasma only†		5	0	5
Hyperimmune plasma	−2 to +10	5	4	4
Hyperimmune plasma	+1 to +10	5	4	3
Hyperimmune plasma	+5 to +10	5	5	3
Hyperimmune plasma	+5* to +10*	5	3	4
Hyperimmune plasma	+8 to +10	5	3	3
No plasma Injected		6	6	0
Probability, ‡ Plasma vs. no plasma			.45	.02
Experiment 2 (*Stock A*):				
Hyperimmune	0 to +14	10	1	10
Hyperimmune plasma	+6 to +14	10	7	7
Normal plasma	0 to +14	10	8	2
Probability,‡ immune vs. normal plasma	(0 to 14)		<.01	<.01
Experiment 3 (*Stock A & B*):				
Stock A, hyperimmune plasma	+5 to +11*,+13*	68	67	28
Stock B, hyperimmune plasma	+6 to +12*,+14*	61	51	23
Stock B, no plasma injected		15	15	2
Probability,‡ immune plasma vs. no plasma (Stock B)			.20	.16

* By intravenous route—all other injections by intraperitoneal route.
† Plasma injections over same period as group below.
‡ Chi square test.

This preliminary experiment indicated that the hyperimmune plasma had a definite effect on the growth and malignancy of transplanted tumors. Though the effect on the incidence of tumors may be questionable, the difference in mortality between the birds treated with plasma and those without such treatment was quite pronounced.

Experiment 2.—In this experiment 30 chicks 3 days of age were inoculated with 1,000 lymphoid tumor cells. Ten of the chicks were injected intraperitoneally with hyperimmune plasma in doses of 1 ml. at 0, 2, and 4 days after inoculation and 2 ml. at 6, 8, 10, 12, and 14 days after inoculation. The second group of 10 inoculated chicks was given 2 ml. of the same plasma on alternate days beginning with the sixth and ending on the 14th day after inoculation. The remaining 10 chicks were injected with plasma collected from adult birds that had previously received no injections and that had showed no pathological lesions. During the experimental period of 21 days, only 1 bird of the 10 that had received immune plasma during the 14 day period developed a tumor and all birds survived; whereas among those that received normal plasma during the same period, 8 birds developed tumors and only 2 survived (Table IV). The majority of chicks, receiving the immune plasma during the 6 to 14 day period developed tumors but most of these also survived. These results confirm those obtained in the first experiment suggesting that the hyperimmune plasma reduced materially the malignancy of the tumor. In addition, there was some indication that the actual incidence of tumors was reduced when the injections began early.

Experiment 3.—The primary purpose of the implants and injections made in chicks listed under Experiment 3 was to produce a number of strain RPL 12 tumor-immune chickens to be used in connection with another phase of this study already reported (4). At 2 days of age all chicks received 500 tumor cells, and at 38 days, 500,000 tumor cells. As a measure to reduce the mortality from these implants, 1.0 cc. of hyperimmune plasma was injected daily from the fifth and sixth to the 13th and 14th days after tumor implantation for stocks A and B, respectively. Fifteen chicks of stock B were also implanted with tumor cells but did not receive any plasma injections and thus served as controls on the effect of the plasma treatment.

During a period of 57 days after the first implantations most of the birds developed tumors at the site of inoculation. The plasma had very little effect on the incidence of tumors, since 83 per cent of the stock B plasma-treated birds, 99 per cent of the stock A birds, and 100 per cent of the birds that did not receive plasma, developed tumors; however, its apparent effect in reducing the malignancy of the tumors was much greater. The rate of survival of the plasma-injected groups was 41 per cent for stock A and 38 per cent for stock B, whereas only 13 per cent of the controls survived.

DISCUSSION

A considerable reduction in the malignancy of tumor transplants, as the results of injection of hyperimmune plasma, was consistently obtained in the three experiments. The effect of the plasma on the growth of the tumor was not so consistent. Results of Experiment 1 indicate that the immune plasma caused some reduction in the incidence of primary tumors although no relation to the number of injections given is apparent. However, in Experiment 2 a reduction in the incidence of tumors was quite pronounced in the group that received immune plasma injections beginning with the day of implantation.

For the 3 experiments the average incidence of tumors in the chicks that received no plasma, or were injected with normal plasma, was 94 per cent. The over-all average for those that received the hyperimmune plasma was 63 per cent. Of those that received immune plasma injections beginning within 1 day after tumor implantation, only 45 per cent developed a tumor. The results thus indicate that not only was there a reduction in mortality among tumor implanted birds as the result of injections of hyperimmune plasma, but there was also a marked reduction in the tumor incidence.

SUMMARY

I. The cytotoxic activity of serum from normal, non-injected chickens and others that had received several injections of lymphoid tumor antigen and normal lymphoid tissue antigen was investigated in a series of 11 experiments.

(a) When lymphoid tumor cells were suspended in normal serum for 24 hours at 20° to 37° C. and then injected into the pectoral muscle, nearly all (93.5 per cent) of the 123 young chicks developed tumors. However, when lymphoid tumor cell antiserum was used instead of the normal serum, only a few (15.7 per cent) of the 127 chicks developed tumors.

(b) The cytotoxic activity of the lymphoid tumor cell antiserum was considerably less when the incubation temperature was reduced to 2° to 7° C. or the incubation period to 2, 3, and 8 hours.

(c) Antiserum produced by the repeated injections of lymphoid tumor cells that had been killed or broken up by rapid freezing and thawing, homogenizing, or heating to 50° C., was also found to be highly toxic to lymphoid tumor cells.

(d) Serum from chickens that had received repeated injections of lymphoid tissue from normal birds had no effect on the growth of lymphoid tumor cells.

(e) Heating lymphoid tumor antiserum to 56°, 61°, or 66° C. for 30 minutes appeared to have no influence on its cytotoxic activity.

The results suggest that an antigen, absent in normal lymphoid tissue but present in the lymphoid tumor cell, provokes the formation of an antibody-like factor in normal adult chickens which is toxic to the tumor cells. This toxicity is expressed by rendering most tumor cells nonviable after an incubation period of 24 hours and suppressing the rate of growth and malignancy of cells that do survive.

II. The effect of lymphoid tumor antiserum (plasma) injections upon the suppression of growth and malignancy of lymphoid tumors implanted in young chicks was studied in three experiments involving 220 birds.

(a) It was found that when plasma from birds hyperimmunized against a lymphoid tumor was injected into birds bearing implants of the same tumor strain, the resulting mortality was much lower than when no plasma was injected or when plasma from normal birds was used.

(b) The incidence of tumor takes was lower when the hosts received injections of hyperimmune plasma.

In summary it may be said that a factor, or factors, found in this lymphoid tumor and antigenically different from constituents of normal lymphoid tissue, is capable of provoking the production of an antibody-like factor in chickens of the same inbred line and that this factor is toxic to tumor cells of the same strain. The effect of this factor is manifested by a partial or complete suppression of the growth of tumor implants. When antiserum is injected into birds with implants, a reduction in mortality and incidence of tumors may be expected.

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The Presence and Significance of Bound Aminoazo Dyes in the Livers of Rats Fed *p*-Dimethylaminoazobenzene*

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The general nature of the carcinogenic process induced by chemicals and radiation can be conceived of as a release of the cell from normal intrinsic and extrinsic growth controls through (a) an activation of a latent virus or the formation of a virus, or (b) a heritable change resulting from reactions between the carcinogen and critical physiological entries. Such concepts may correspond to different aspects of the same process. However, regardless of the correct explanation, an objective that appears reasonably accessible at the present in the study of the chemical carcinogens is the identification of the compound or set of compounds directly concerned in the initiation of the carcinogenic process. Thus, the *parent* carcinogen (compound administered) might be converted in the body to a derivative which could be properly considered as the *primary* carcinogen. Even a partial realization of this objective should enable a more logical attack to be made on the mechanism of the carcinogenic process. Thus the character of the initial phases in the process might become more evident once the properties of the primary carcinogen or carcinogens were known.

The identification of the primary carcinogen(s) has been the immediate objective of our studies on the hepatic carcinogen, *p*-dimethylaminoazobenzene. As a working hypothesis, we have considered that at least one of the primary carcinogens is either *p*-dimethylaminoazobenzene itself or its metabolite, *p*-monomethylaminoazobenzene, a mixture of these dyes, or a compound very closely related to them (14). Our studies on the metabolism of *p*-dimethylaminoazobenzene (10, 16) and the effect of structure on its activity (13, 15) support this hypothesis.

Recently we have observed that the livers of rats fed *p*-dimethylaminoazobenzene contain aminoazo dyes bound tightly to a cellular constituent, presumably protein. This paper contains a description and an assessment of this finding.

PRELIMINARY OBSERVATIONS AND METHODS

In the course of other studies we prepared protein precipitates from the perfused livers of rats fed various diets. When acid precipitants, *e.g.*, trichloroacetic and sulfosalicylic acids, were used the liver protein from normal rats was practically white, whereas that from rats fed *p*-dimethylaminoazobenzene was pink. Our colleague, Dr. W. C. Schneider, brought this fact to our attention several months before the start of this work, but at that time we supposed the color to be due to the free aminoazo dyes known to be present in such livers (16). When the pink precipitates were made alkaline, they turned light yellow; on acidification the pink color returned. The reversible change from pink to yellow in acid and basic media is a useful test for the probable presence of aminoazo dyes in the tissues of rats fed such dyes. The pigment was also irreversibly reduced by $Na_2S_2O_4$ so that the color of the precipitate resembled that from normal rats. This property indicated that the pigment was not a colored quinone. Furthermore, no pink color was noted with acid precipitates from the livers of rats fed any one of the possible or known monophenyl metabolites of *p*-dimethylaminoazobenzene (10, 14, 28), *viz* *p*-phenylene diamine, *N*-methyl-*p*-phenylene diamine, *N*,*N*-dimethyl-*p*-phenylene diamine, *p*-aminophenol, *o*-aminophenol, or aniline. Recovery studies showed that precipitates of normal liver protein made in the presence of 10 per cent trichloroacetic acid strongly adsorbed the acid forms of *p*-dimethylaminoazobenzene, *p*-monomethylaminoazobenzene, and *p*-aminoazobenzene or their *p'*-hydroxy derivatives; successive washings with fresh acid extracted the dyes very slowly. Normal liver proteins precipitated by 10 per cent sulfosalicylic acid adsorbed less added dye and the adsorbed dye could be extracted from the precipitate with 4 or 5 washings with this

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acid. The adsorbed aminoazo dyes were readily removed from normal liver protein precipitates prepared with either acid by washing with ethanol or acetone. However, the acid precipitates made from the livers of rats fed *p*-dimethylaminoazobenzene were still pink after many washings and the dye was not removed by washing with ethanol or acetone. These facts suggested that aminoazo dye tightly bound to the crude protein was present in the livers of rats fed *p*-dimethylaminoazobenzene.[1]

Preparation and composition of crude liver protein precipitates.—In subsequent work heat coagulation was employed in preference to acid precipitants to minimize the adsorption by proteins of the aminoazo dyes known to be present in livers from dye-fed rats and to give preparations free from acid precipitants. The following method was used routinely: Rat livers were perfused *in situ* through the vena cava with 50 ml. of 2 per cent sodium citrate at a hydrostatic pressure of about 15 cm. of water. A gentle massage of the liver was employed to facilitate the perfusion. After the excised liver was minced, 4 gm. samples were homogenized (22) in 16 ml. of water, 20 ml. of 1 *M* sodium acetate buffer, pH 5, were added, and the protein was precipitated by boiling for 3 minutes. After centrifugation the coagulum was washed once with 20 ml. of acetate buffer and twice with 20 ml. portions of 95 per cent ethanol. Only negligible amounts of protein could be precipitated from the supernatant solution or acetate washing by the addition of trichloroacetic acid. It was found convenient to resuspend the precipitates with a homogenizer pestle that fitted the centrifuge tubes quite loosely. The wet precipitates, wrapped individually in quantitative filter paper, were extracted at approximately 60° C. with 95 per cent ethanol in a Soxhlet extractor for 48 hours to remove free aminoazo dyes and lipids. They were then transferred to small beakers, dried to light powders over sulfuric acid *in vacuo*, and stored in tightly closed vials. The dry powders represented 10.4 to 14.0 per cent (av. = 11.8) by weight of the original fresh liver.

The liver powders contained about 1 per cent of glycogen, about 1 per cent of oily matter extracted by boiling for 24 hours with chloroform, 14.9 to 15.9 (av. 15.3) per cent of nitrogen (Kjeldahl), 0.74 to 1.14 (av. 0.90) per cent of phosphorus (Fiske-Subbarow), and about 2.4 per cent of ash when barium acetate was used to fix the excess anions.[2] Nearly all of the ash determined in this manner could be accounted for in terms of the phosphorus as pyrophosphate. These data suggest that the preparations contained approximately 97 per cent of a mixture of protein and nucleoprotein.

Liberation of the bound dyes.—Attempts to liberate the dyes from the crude protein by mild procedures that left the protein essentially intact were unsuccessful. The content of dye in the powder (as determined by the method described below) was the same before and after extraction of 200 mgm. of the dry powder with 25 to 50 ml. of boiling chloroform, peroxide-free ethyl ether, 1:1 ethyl ether-ethanol, ethanol, acetone, benzene, pyridine or n-butanol for 24 hours. The incubation of the powder for 24 hours at room temperature in a strong detergent (1 per cent pentrant 7, Carbide and Carbon Corp.) also did not liberate any dye extractable with ethanolic ethyl ether.

Furthermore, removal of the nucleic acids from the liver powders with hot trichloracetic acid (25) did not alter significantly the content of bound dyes with reference to the original liver protein. For this experiment the crude protein was prepared from 4 gm. samples of pooled livers from dye-fed rats according to the procedure given above but with the omission of the exhaustive ethanol extraction. Two of the samples were kept as controls and each of 2 other samples was suspended in 100 ml. of 5 per cent trichloroacetic acid and heated at 90° C. for 15 minutes. After centrifugation the residual protein was washed once with 25 ml. of cold 5 per cent trichloroacetic acid and twice with 20 ml. of ethanol. All 4 samples were then extracted with hot ethanol for 48 hours and dried. The dry weight of the treated powders was 84 per cent of the weight of the control powders and the phosphorus contents were 0.20 and 1.14 per cent for the treated and control samples respectively. Similarly the desoxyribose nucleic acid and ribose nucleic acid contents, determined by the method of Schneider (25), decreased from 4.29 and 3.24 per cent to 0.18 and 0.16 per cent respectively in the control and treated preparations.[3] However, the contents of total bound dye were very similar: E values (*see* below) of 0.182 and

[1]Since the submission of this paper further proof has been obtained that protein bound dyes actually exist *in vivo* and are not artifacts. Dialysis of the soluble proteins of liver homogenates (the "$CaCl_2$ supernate" of W. C. Schneider, *J. Biol. Chem.*, **166**:595. 1946) against isotonic KCl buffered with 0.005 *M* phosphate to pH 7.3 at 0-3° C. for 1 or 2 days did not alter the bound dye content of the proteins. Furthermore, a 3-fold dilution of the soluble proteins prior to coagulation did not change the amount of protein obtained or its content of bound dye.

[2]Several of these analyses were kindly performed by Mrs. H. N. Kingsley and Dr. R. K. Boutwell of this laboratory.

[3]Our thanks are due to Mr. J. M. Price for these analyses.

0.144 per 100 mgm. of dry powder or 0.172 and 0.163 per gm. of original fresh liver for the treated and untreated samples respectively. Thus, since the proteins from powders heated with trichloracetic acid contained 106 per cent as much bound dye as the original proteins it appears that the bound dyes are contained in the protein residue—Fraction IV of Schneider (25).

In contrast to the direct extraction of the liver powder, degradation of the powder with trypsin liberated dye which could be extracted from the digests with ethanol-ethyl ether. For these experiments 100 mgm. samples of the crude protein were dispersed in 5 ml. of 1 *M* NH_4OH-NH_4Cl, pH 8.6, and incubated at 37° C. in the presence of benzene as a preservative. Some tubes were kept as controls without further additions; to the others 10 mgm. of purified trypsin in 1 ml. of buffer were added on the first and third days. After 1 week of digestion only 10 per cent of the original protein could be precipitated by 10 per cent trichloroacetic acid. At this time 4 ml. of 11 *N* KOH and 4 ml. of ethanol were added to each tube and the digests were extracted immediately with ethyl ether (*see* method below). Only negligible amounts of dye (E = 0.010 per 100 mgm.) were obtained from the control tubes but appreciable amounts (E = 0.126 per 100 mgm.) were extracted from those tubes treated with trypsin.

However, hydrolysis with alkali under optimal conditions liberated about 2½ times as much dye as tryptic hydrolysis and was found to be the most satisfactory method of liberating the bound dye for analysis. As shown in Table I the amounts of the bound dyes obtained by hydrolysis of the powder in ethanolic KOH increased markedly when the temperature was raised from 25° to 80° C. However, even at 80°, the amount of extractable dye increased with time up to 92 hours. Thus, if the amount of bound dyes liberated by hydrolysis after 92 hours be designated as 100 the amounts obtained after 12, 16, 20 and 44 hours of hydrolysis were 80, 84, 87, and 96 per cent respectively. The increasing E values obtained with increasing time appeared to be due to an increased liberation of extractable dyes rather than to a change in the character of the liberated dyes; when a concentrate of the isolated dyes from 20 hour hydrolyses was hydrolyzed in ethanolic KOH for 20 hours, as in the method below, the E value of the recovered dye was 90 per cent of the E value prior to hydrolysis. Continuing the hydrolysis for still longer periods caused some destruction; only 82 per cent as much dye could be extracted after 116 hours as was obtained at 92 hours.

Hydrolysis in sealed 16 × 150 mm. tubes appeared promising at first, but the maximum values obtained at temperatures of 100° to 125° C. after periods of 1 to 3 hours were only 80 per cent as high as those obtained after 20 hours of hydrolysis at 80° C.; after the maximum values were reached, destruction was fairly rapid. At 80° C. the amount of dye obtained and the rate of liberation were approximately equal in open 25 × 200 mm. test tubes and in sealed 16 × 150 mm. test tubes as long as the tubes were sealed close to the end. When the tubes were sealed leaving less space above the liquid, lower and inconsistent values were obtained.

TABLE I: THE EFFECT OF TIME AND TEMPERATURE ON THE LIBERATION OF THE BOUND DYES WITH ETHANOLIC KOH FROM LIVER POWDER PREPARED FROM RATS FED *p*-DIMETHYLAMINOAZOBENZENE

(Values expressed as E/100 mgm.)*

Time, hrs.	Basal diet + dye			Basal diet	Bound dye
	25° C.	60° C.	80° C.	80° C.	
1	0.025	0.105	0.148		
2	0.035	0.120	0.180		
3	0.049	0.135	0.206		
6	0.059		0.234		
12			0.304	0.040	0.264
16			0.318	0.040	0.278
20			0.338	0.050	0.288
44			0.392	0.076	0.316
92			0.434	0.104	0.330
116			0.384	0.112	0.272

* $E = \log_{10} I_0/I$ of acid-ethanol solutions described under *Estimations of the bound dyes.*

Properties of the bound dyes.—The bound dyes liberated by alkaline hydrolysis can be fractionated by extraction with petroleum ether and ethanolic ethyl ether respectively into non-polar and polar dyes. The non-polar dyes have been further separated by chromatography on an aluminum oxide column into two fractions, which have been identified as *p*-monomethylaminoazobenzene and *p*-aminoazobenzene (12); no *p*-dimethylaminoazobenzene could be detected. The identity of these compounds was established by means of mixed chromatograms with the authentic compounds and by their absorption spectra in 7 *N* HCl. Furthermore, *p*-monomethylaminoazobenzene and *p*-aminoazobenzene were isolated chromatographically from a digest of 8 gm. of liver powder, reduced to their constituent amines with Sn-HCl, and the amines were then determined by a relatively specific colorimetric method (14). In this method primary aromatic amines form Schiff bases with sodium β-naphthoquinone sulfonate which have characteristic solvent properties and absorption spectra; equimolar amounts of amines with properties identical with aniline and monomethyl-*p*-phenylene diamine and aniline and *p*-phenylene diamine were found after the reduction of the dyes.

Previous reports (16) have shown that *p*-dimethylaminoazobenzene, *p*-monomethylaminoazobenzene, and *p*-aminoazobenzene were present in the livers of rats fed either methylated compound in a relatively free form. Thus, although cold ethanolic KOH was employed in the quantitative determination of the free dyes (12) to destroy the tissue structure, eliminate emulsions, and help force the dyes into the solvent, the preliminary digestion was not essential; in spite of almost unmanageable emulsions the dyes could be extracted with petroleum ether directly from fresh liver homogenates at pH 7. However, the non-polar dyes isolated from the liver powder must have been bound since (a) no dye could be extracted with petroleum ether after suspending the powder in cold ethanolic KOH for 1 to 2 hours (12) and (b) the immediate extraction of a solution of the powder in hot ethanolic KOH after rapid cooling yielded only 10 per cent of the non-polar dye that could be obtained after hydrolysis for 20 hours at 80° C. After 20 hours of alkaline hydrolysis at 80° C., the dye extracted with petroleum ether gave E values which were 6 to 13 per cent (average 8.8), as great as the E value for the total dyes extracted with ethanol-ethyl ether. About 0.2 μgm. of bound *p*-aminoazobenzene and 0.5 μgm. of bound *p*-monomethylaminoazobenzene per gm. fresh weight were found in the livers of rats fed *p*-dimethylaminoazobenzene for 1 month; in the free form these livers contained about 0.2 μgm. of *p*-dimethylaminoazobenzene, 0.2 μgm. of *p*-monomethylaminoazobenzene, and 1.5 μgm. of *p*-aminoazobenzene. When any of these three compounds was added to the liver powder from normal rats, 90 to 102 per cent could be recovered after 20 hours of alkaline hydrolysis at 80° C.

The major share of the bound dye obtained by the hydrolysis of the liver powder had a highly polar character, and was readily extracted only with a mixture of ethanol and ethyl ether or, slowly, with amyl acetate. After removal of the solvent, most of the extracted dye was soluble in strong hydrochloric acid, but ethanolic hydrochloric acid was necessary for complete solution of the crude polar dye residue. The structure of this fraction is unknown and in the present paper it is termed the "polar bound dye." Its relationship to the non-polar bound dyes is under investigation.

Estimation of the bound dyes.—In the method adopted for the quantitative analysis of total bound dyes, 50 mgm. samples of powder were digested in a mixture of 2 ml. of ethanol and 5 ml. of 4.5 *N* KOH for 20 hours in 25 × 200 mm. pyrex test tubes equipped with finger condensers. The tubes were heated in a mineral oil bath kept at 80° C. After digestion the samples were cooled and 2 ml. of ethanol, 4 ml. of water, 4 ml. of 11 *N* KOH, and 10 ml. of peroxide-free ethyl ether were added to each tube. Then the digests were stirred for 5 minutes on a multiple stirring assembly with spiral glass stirrers. The ethereal layers were transferred to clean 25 × 200 mm. test tubes through a capillary pipette operated by air pressure (11), and the aqueous residues were then re-extracted with 6 cc. of a 1:5 ethanol-ethyl ether mixture. The combined ethereal layers were evaporated to about 5 cc. in a water bath at atmospheric pressure and then taken to near-dryness (<0.1 ml.) *in vacuo*: smooth boiling was obtained with the aid of inverted glass capillaries (boiling point tubes) sealed at the top and 2 to 3 mm. from the bottom. The residues were dissolved in 2 ml. of ethanol which was delivered evenly over the inside of the tubes and 2½ ml. of 7 *N* HCl were added to develop the colors. The short ends of the capillaries were crushed with glass rods, 1 ml. of light petroleum ether was added to each tube, and the tubes were shaken to cause any insoluble residue to collect at the interface. The samples were then poured into matched 13 × 100 mm. pyrex culture tubes, centrifuged, and read in a Cenco-Sheard spectrophotelometer at 520 mμ. 15 to 30 minutes after the addition of the acid. All values were expressed arbitrarily as E $(\log_{10} I_0/I)_{520}$ mμ. per 100 mgm. dry powder under the conditions described.

For the determination of bound *p*-monomethylaminoazobenzene and *p*-aminoazobenzene the alkaline digests of the powders were diluted with ethanol, water, and KOH as described above and then extracted twice with 15 ml. of petroleum ether; the combined extracts were then taken to dryness *in vacuo* and the residues transferred to colorimeter tubes with small volumes of fresh petroleum ether. Three milliliters of 7 *N* HCl were added to each tube and the dyes were extracted into the acid with vigorous shaking. Since only small quantities of these dyes were present they were generally estimated together from the E values of these solutions read at 505 mμ. The amounts of polar bound dye in the digests were then determined as described above after 4 ml. of ethanol were added to each digest to compensate for the losses occasioned by the preliminary extractions with petroleum ether. Since the major share of the total bound dye was found to be polar bound dye the analyses reported in this paper, unless otherwise specified, were estimations of total bound dye.

The results of replicate analyses of 25 to 50 mgm. samples for total bound dye always agreed within ± 6 per cent of their mean and at least 70 per cent

of the values were within ± 3 per cent of this mean. The bound dye contents of duplicate powders from the same liver homogenate also agreed within the limits of the analytical method. When 100 mgm. of powder were hydrolyzed in the same volumes of ethanol and KOH, the values obtained were about 20 per cent low. Similar difficulties have been experienced in preliminary experiments on the liberation of the bound dyes from larger quantities of powder; work on this point is being continued. When the isolated polar bound dye in amounts equivalent to E values of about 0.150, alone or in the presence of 50 mgm. of liver powder obtained from normal rats (designated hereafter as basal liver powder), was carried through the digestion and extraction procedures, about 90 per cent was recovered.

Acid-ethanol solutions of the polar bound dye obeyed Beer's law in the range E = 0.033 to 0.639. When basal liver powders from perfused rats were analyzed, the acid-ethanol solution was light yellow in color and gave an E value averaging 0.050 per 100 mgm. when read 15 to 30 minutes after the acid was added. Presumably comparable amounts of interfering materials such as residual blood (*see* below) were present in the extracts from dye-containing powders; except where specified, this correction for non-specific absorption was applied to all determinations of bound dye reported here. If peroxide-free ether was used for the extraction, the pink color of the acid-ethanol solution was stable indefinitely but if ether containing peroxides was used, the pink color gradually faded to yellow.

Feeding methods.—Adult animals were used in the experiments described below and they were housed in screen-bottom cages in groups of 4 to 8 when fed *ad libitum* and in pairs of equal size when fed restricted amounts of diet. In the experiment on species variation all of the animals were fed a grain diet (3) with supplements of greens. In the other experiments the following semisynthetic basal diet (18, 19) was used: crude casein, 12; Vitab rice bran concentrate, 2; salts, 4; cerelose, 77; and corn oil, 5. Riboflavin was added to adjust the concentration of this vitamin to 0.5, 1.0, 10.0, or 20.0 mgm. per kgm. of diet. Each rat also received 1 drop of halibut liver oil a month. When 0.06 per cent *p*-dimethylaminoazobenzene is added to this diet and fed to rats, a high incidence of liver tumors results after 4 months in those animals receiving low levels of riboflavin.

RESULTS

Bound dye content of rat tissues.—The tissues of 2 rats fed *p*-dimethylaminoazobenzene in the semisynthetic diet (0.5 mgm. riboflavin per kgm.) for 2 months were analyzed to determine the distribution of bound dye; the tissues of similar animals fed the basal diet were used as controls. Blood samples were withdrawn by heart puncture, and the whole heparinized animals were then perfused through the vena cava below the renal veins with 300 ml. of isotonic sodium citrate (2). The small intestine, liver, spleen, heart, kidneys, lungs, and the skeletal muscle from each pair of rats were pooled, minced, and homogenized as well as possible. The crude protein from these organs and from the plasma and the red blood cells was then prepared in the usual manner and the dry powders were analyzed for bound dye as described above. Most of the tissues from the rats on the basal diet gave light yellow acid-ethanol solutions with E = 0.030 to 0.058 per 100 mgm. (Table II). The spleen and red blood cells, however, gave much larger absorptions of 0.190 and 0.332; these solutions were red-brown in color with maximum absorption at 490 mμ. Furthermore, unlike the bound dye, the intensity of the color increased with time so that the absorption was about 30 per cent greater 3 hours after adding the acid and ethanol than it was 20 minutes after these additions and 100 per cent greater 24 hours later. The interference was probably due to hemoglobin derivatives and appeared to be responsible for most of the low absorption of the other tissues since the acid-ethanol solutions of these tissues also absorbed more strongly at 490 mμ. than at 520 mμ. and the intensity of absorption increased with time. For instance, normal perfused rat liver gave

TABLE II: THE LEVEL OF TOTAL BOUND DYE IN THE TISSUES OF RATS FED *p*-DIMETHYLAMINOAZOBENZENE

(Average of 2 rats; diets fed for 2 months)

	E/100 mgm. powder			Color of acid-ethanol solution	
Tissue*	Basal diet + dye	Basal diet	Bound dye†	Basal diet + dye	Basal diet
Liver	0.330	0.050	0.280	Pink	Light yellow
Lung	0.044	0.030	—	Light yellow	" "
Heart	0.088	0.058	—	" "	" "
Kidney	0.074	0.054	—	" "	" "
Skeletal muscle	0.038	0.038	—	" "	" "
Small intestine	0.034	0.046	—	" "	" "
Blood plasma	0.078	0.018	0.060	Light pink	Nearly colorless
Red blood cells	0.356	0.332	—	Red-brown	Red-brown
Spleen	0.304	0.190	—	"	"

* The blood was removed by perfusion of the whole rats.

† Only in the case of liver and blood plasma do the differences in absorption and color of the extracts from tissues of normal and dye-fed rats indicate the presence of bound dye. In the case of the other organs the small differences can probably be ascribed to differences in the amount of residual blood and other pigments. The spleens of rats fed the azo dye were engorged with red cells and their degradation products which were not removed by perfusion.

a solution with an absorption of E = 0.050, 20 to 30 minutes after the addition of the acid and 0.064, 90 minutes after the acid was added; non-perfused rat liver powder gave solutions with E equal to or greater than 0.078 twenty minutes after the addition of the acid.

Like the tissues from the rats fed the basal diet, the lungs, heart, kidneys, skeletal muscle, and small intestine from the dye-fed rats had low absorptions of the same order of magnitude as their controls; with these tissues the small differences between the basal and dye-fed animals did not appear to be significant and may have been related to the amounts of blood left in the tissues after perfusion. The spleen and red blood cells again gave red-brown solutions which absorbed strongly. Since the E values for the red blood cells from normal and dye-fed rats were nearly equal, no appreciable quantity of bound dye could have been present. The spleens from the dye-fed rats gave solutions with E = 0.304 while normal spleen powders absorbed less strongly. However, the spleens of rats fed the azo dye were enlarged and dark and are known to be engorged with blood cells and their breakdown products (5, 21); hence the greater absorption was probably due to an increased blood content.

The liver, on the other hand, gave a pink acid-ethanol solution with E = 0.330 per 100 mgm. of powder as compared to E = 0.050 for the basal powder. The E value increased to 0.340 in 90 minutes; this was approximately the same absolute increase as was obtained with the basal powder. The plasma also appeared to contain a small amount of bound dye since the plasma powder from the dye-fed rats gave a light pink solution with E = 0.070 per 100 mgm. whereas the E value for the plasma from rats on the basal diet was 0.018. This observation is of interest in view of the prominence of the liver in the synthesis of the plasma proteins (8).

Free and bound aminoazo dyes in the livers of several species.—In order to compare the levels of free and bound aminoazo dyes in the blood and livers of various species several rats, mice, rabbits, cotton rats, guinea pigs, and chickens were fed the grain diet containing 0.06 per cent of *p*-dimethylaminoazobenzene. The grain diet was used in preference to the semi-synthetic diet which we generally use for rats since guinea pigs and rabbits will not consume adequate amounts of the purified diet. After 2 months some of the animals were used for analysis of the free aminoazo dyes in the blood and liver while the livers of others were analyzed for bound aminoazo dye in the usual manner; the livers were perfused in each case. The livers of rats and mice contained about the same amounts of the free aminoazo dyes, whereas the blood of mice contained considerably more *p*-aminoazobenzene than that of rats and in addition small amounts of *p*-dimethylaminoazobenzene which is seldom detectable in rat blood (Table III). Little or no dye was found in the blood of cotton rats and rabbits while only low levels of *p*-aminoazobenzene were found in the livers of rabbits; larger amounts of *p*-aminoazobenzene and traces of the methylated dyes were detected in the livers of cotton rats. The livers and blood from chickens contained appreciable amounts of *p*-aminoazobenzene, but only traces of the dimethyl or monomethyl compounds were present in the liver.

As seen from Table III a correlation can be made between the levels of the free methylated aminoazo dyes in the liver and the susceptibility of the species to the induction of liver tumors by *p*-dimethylaminoazobenzene. However, the amount of bound dye in the liver appears to correlate even better with the susceptibility to the dye. Bound dye has been detected only in the livers of rats and mice, the two species in which tumors have been induced by *p*-dimethylaminoazobenzene. Further, the livers of mice, which develop tumors more

TABLE III: THE LEVELS OF FREE AND BOUND AMINOAZO DYES IN THE LIVERS AND BLOOD OF ANIMALS FED *p*-DIMETHYLAMINOAZOBENZENE

(Averages of 2 to 5 animals diets fed for 2 months)

	Liver (perfused) free aminoazo dyes μgm./gm. fresh wt.			Blood free aminoazo dyes μgm./ml.		Bound aminoazo dye E/100 mgm. liver powder	Susceptibility to induction of liver tumors with DAB	
	DAB*	MAB*	AB*	DAB	AB			
Albino rat (Sprague-Dawley)	0.32	0.19	1.83	Not detectable	13.4	0.236	High	(5,24)
Albino mouse (abc)	0.17	0.11	2.20	0.46	83.0	0.070	Low	(5,6,24)
Guinea pig†	<——— Not detectable ———				>	Not detectable	None	(21)
Rabbit†	Not detectable		0.20	Not detectable		"	Very low	(5)
Cotton rat†	0.09	0.01	1.54	Not detectable	1.55	"	Very low (Present authors)	
Chicken (white leghorn)	0.04	0.04	0.84	"	11.9	"	None	(5)

* DAB = *p*-dimethylaminoazobenzene. MAB = *p*-monomethylaminoazobenzene. AB = *p*-aminoazobenzene.
† Strain unknown.
‡ The final acid-ethanol solutions were light yellow and gave uncorrected E values of 0.062—0.068 (normal rat liver = 0.050).

slowly than livers of rats (5, 6, 24), have only 30 per cent as much bound dye as does rat liver. Chickens (5), guinea pigs (21), and rabbits (5) are highly resistant to tumor induction by *p*-dimethylaminoazobenzene and their livers did not contain detectable amounts of bound dye. No bound dye was found in the livers of cotton rats; in this laboratory no gross pathological changes in the liver of this species have been found after 8 months of dye-feeding. Apparently the differences in the levels of free and bound aminoazo dyes in the tissues of these species are not due to variations in the amount of dye absorbed from the tract. About 50 per cent of the dye can be accounted for in the urine of the rodents as conjugated *p*-aminophenol and *p*-phenylene diamine and small amounts of other metabolites (10); this would indicate that about the same proportion of the ingested dye was absorbed in each case.

The level of bound dye as a function of dietary riboflavin and the duration of feeding.—To determine the level of bound dye after various periods of dye-feeding, 2 groups of 21 rats were fed 0.06 per cent of *p*-dimethylaminoazobenzene in the semisynthetic diet containing 1 or 10 mgm. of riboflavin per kgm. of diet. The lower level of riboflavin allows a high incidence of liver tumors to develop while 10 mgm. per kgm. prevents the development of hepatomas by 6 months (4, 17, 19). Three rats from each group were sacrificed at periods of 4 days to 20 weeks from the beginning of the experiment. After only 4 days on the dye the liver powder from the rats on the low riboflavin diet gave an E value of 0.080 per 100 mgm., although 4 weeks were required before the maximum value of 0.350 was reached (Fig. 1). After 4 weeks the level of bound dye dropped slowly so that the level at 20 weeks (E = 0.176) was only slightly higher than at 1 week. A curve of similar shape was obtained from analyses of the liver powders from the rats on the high riboflavin diet but the maximum level of bound dye occurred somewhat later at about 5 to 6 weeks. Furthermore, the quantities of bound dye were only 41 to 79 per cent as great on the high riboflavin diet as on the low riboflavin diet at each point from the first to the 20th week. The differences between the livers of rats from the same group killed at a given time were sometimes considerable, but after the first week the ranges for the livers of rats, on the low and high riboflavin diets did not overlap appreciably except at the 8 week analysis. The

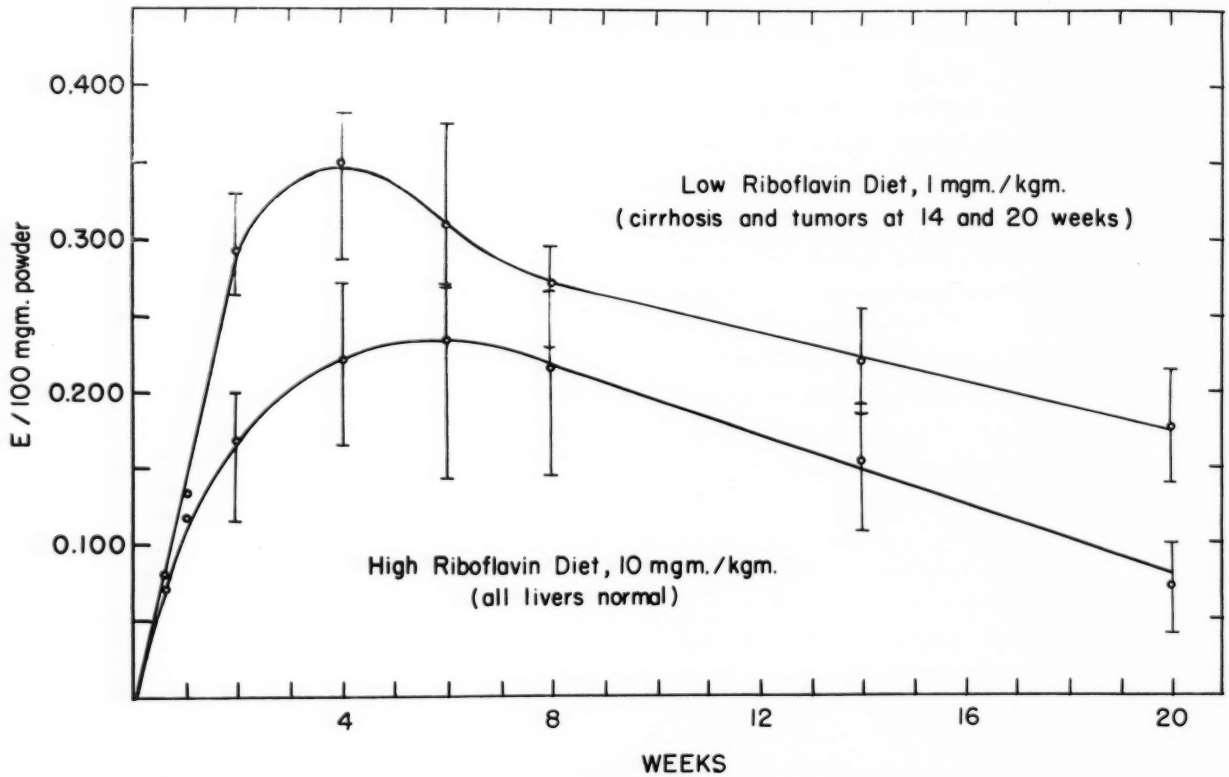

FIG. 1.—Levels of total bound dye in livers of rats fed *p*-dimethylaminoazobenzene continuously in diets low and high in riboflavin. Each point and vertical bracket represent average and range respectively of 3 rats. The points at 6 weeks were obtained in a separate experiment.

bound dyes from the livers of these rats always contained approximately the same ratio of non-polar and polar dyes regardless of the level of riboflavin or the period for which *p*-dimethylaminoazobenzene was fed.

In a second series, groups of 4 rats were fed the semi-synthetic diet containing 0.06 per cent of *p*-dimethylaminoazobenzene with 1, 10, or 20 mgm. of riboflavin per kgm. of diet; the rats were restricted to 8 gm. of food per day. After 6 weeks all the animals were killed and the level of bound dye was determined. The E values for the rats given 1 or 10 mgm. of riboflavin per kgm. were 0.310 and 0.246, respectively, and agreed well with those obtained by interpolation from the curve in Fig. 1. The level of bound dye in the livers of rats receiving 20 mgm. of riboflavin per kgm. of diet was equal to 0.234 at 6 weeks; this was only slightly lower than the level of dye in the livers of rats fed half this level of the vitamin.

Since earlier experiments demonstrated that the addition of 10 mgm. of riboflavin per kgm. of diet strongly inhibited the carcinogenic activity of *p*-dimethylaminoazobenzene, it was important to determine whether high amounts of this vitamin decreased the level of bound dye by facilitating the degradation of the complex once formed, by acting prior to the binding of the dye, or by a combination of these means. In an attempt to differentiate these two modes of action the semi-synthetic diet containing 0.5 mgm. of riboflavin per kgm. and 0.06 per cent of *p*-dimethylaminoazobenzene was fed *ad libitum* for 5 weeks to a group of 22 rats. Four rats were then killed, and the level of bound dye in the liver was determined. Nine rats were given the basal diet containing 0.5 mgm. of riboflavin per kgm. and 9 were injected intraperitoneally once with 500 μgm. of riboflavin and then fed the basal diet containing 10 mgm. of this vitamin per kgm. To maintain the rats at approximately their original weights they were restricted to 6 to 7 gm. of the basal diets per day; this was necessary to minimize changes in the amount and composition of the liver protein due to growth or starvation. Three rats from each of the 2 groups were killed after 4, 7, and 14 days on the basal diets. Expressing the level of bound dye at zero time as 100 per cent the levels had dropped to 69 and 10 per cent after 4 and 7 days on the low riboflavin diet. The bound dye in the livers of rats fed the high riboflavin basal diet fell even faster so that only 47 and 7 per cent as much bound dye was present after 4 and 7 days as at the beginning. No detectable quantity of bound dye was left in the livers after 14 days on the basal diets. In another experiment the livers of rats fed the low riboflavin basal diet contained 69 per cent as much bound dye after 4 days on the basal diet as was present when the dye-feeding was stopped; whereas the livers of rats on the high riboflavin basal diet had only 43 per cent as much bound dye as that present initially. These data suggest that riboflavin acts, at least in part, by aiding the destruction of the bound dye after it is formed, but cannot indicate whether or not riboflavin may also act before the complex is formed.

Bound dyes in the livers of rats fed p-dimethylaminoazobenzene, p-monomethylaminoazobenzene, and p-aminoazobenzene.—Liver powders were prepared from rats fed 0.060 per cent of *p*-dimethylaminoazobenzene, 0.056 per cent of *p*-monomethylaminoazobenzene or 0.106 per cent of *p*-aminoazobenzene in the semi-synthetic diet containing 0.5 mgm. of riboflavin per kgm. for 6 weeks. The *p*-aminoazobenzene was fed at twice the molar level of the methylated compounds since it gave rise to such low levels of polar bound dye. The powder from rats fed *p*-monomethylaminoazobenzene was found to contain bound *p*-monomethylaminoazobenzene and *p*-aminoazobenzene at levels comparable to those given above for the powders from rats fed *p*-dimethylaminoazobenzene. In each case about 6 to 8 μgm. of dye per gm. of powder was found in the petroleum ether extracts of the hydrolysates; about ⅓ of this amount was *p*-aminoazobenzene. Only *p*-aminoazobenzene was found in the petroleum ether extracts of hydrolysates of liver powder from rats rats fed *p*-aminoazobenzene. On the average 7.2 μgm. of bound *p*-aminoazobenzene per gm. of powder were found in these livers; this level is about four times that found in the livers of rats fed the methylated dyes at half the molar level used with *p*-aminoazobenzene.

The absorption spectra of the polar bound dyes isolated from the liver powders of rats fed *p*-dimethylaminoazobenzene or *p*-monomethylaminoazobenzene are shown in Fig. 2. The dyes were released from 50 mgm. of powder, taken up in acid-ethanol and read in matched 13 × 100 mm. pyrex culture tubes in a Beckman DU Spectrophotometer. In each case the curves were corrected for the absorption given by basal powder. For comparison, similar curves are shown for *p*-dimethylaminoazobenzene, *p*-monomethylaminoazobenzene, and *p*-aminoazobenzene.

The polar bound dyes from the rats fed either methylated dye absorbed maximally at 523 mμ. and the similarities between the spectra of the bound dyes and their precursors offer additional evidence that the former are aminoazo dyes. The shape of the 2 curves indicates that the polar bound

dyes in these two instances are the same; this is supported by the fact that the ratio of the E values of these two curves varies only from 1.12 to 1.14 in the region of 500 to 550 mμ. These similarities parallel our earlier observations that the free monomethyl and dimethyl dyes are both present in the livers of rats fed either dye (16) and are of equal carcinogenic potency (9, 13).

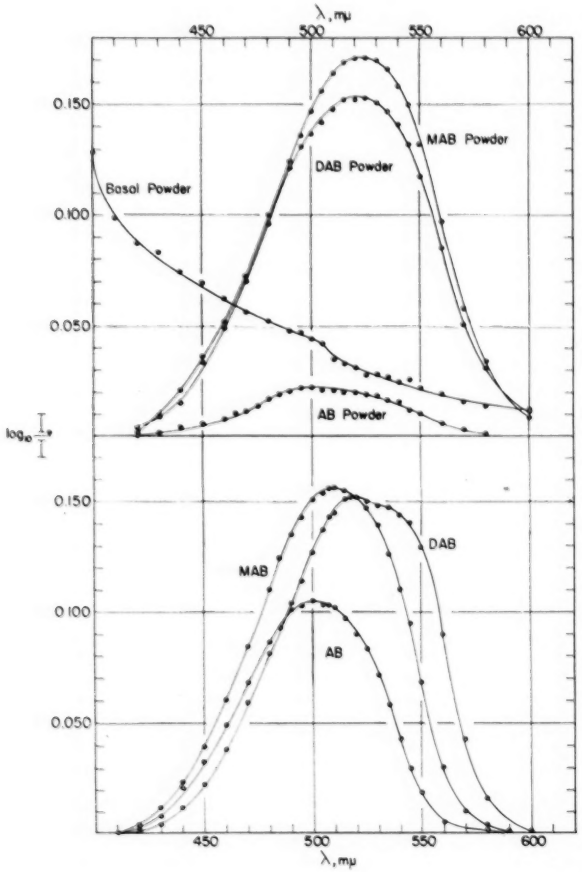

FIG. 2.—Absorption spectra of polar bound dyes present in liver powders from rats fed either *p*-dimethylaminoazobenzene (DAB) or *p*-monomethylaminoazobenzene (MAB). Curve given for powder from rats fed *p*-aminoazobenzene (AB) represents spectrum of total bound dyes. Each spectrum has been corrected for absorption given by basal powder. DAB and MAB were fed at equimolar levels of 0.06 and 0.056 per cent respectively; AB was fed at twice this molar level, *i.e.*, 0.106 per cent. Absorption spectra of DAB, MAB, and AB are given for comparison. All the spectra were determined in acid-ethanol; the extracts from 50 mgm. of liver powder were dissolved in 4.5 ml. of solvent while 3.5×10^{-6} molar solutions of the other dyes were used. Effective cell thickness = 1.09 cm.

The spectrum given in Fig. 2 for the extract from the liver powder of rats fed *p*-aminoazobenzene represents the total bound dye in 50 mgm. of powder. The extract absorbed maximally at 500 mμ. but the curve was broader than that for *p*-aminoazobenzene. Further, although the rats were fed twice as much dye, the maximum E values for this liver powder was only 1/6 that of the liver powders from rats fed the methylated dyes. About ½ of the E values given in corrected curve are attributable to a dye extractable only with ethanol-ethyl ether; the similarity of the curve to that of its precursor, *p*-aminoazobenzene, suggests a similarity in structure although the correction for the basal powder was large in this instance. In this connection it is of interest that the methylation of *p*-aminoazobenzene *in vivo* has never been demonstrated (9, 16), and this compound is, for practical purposes non-carcinogenic (5, 9, 13). However, *p*-aminoazobenzene is a metabolite of the methylated dyes (16) and it is probable that a very low level of polar bound dye derived from *p*-aminoazobenzene is present in the livers of rats fed the carcinogenic methylated dyes.

Since the E value per unit weight for the polar bound dyes is unknown, it is not possible to state the actual amount of polar bound dye present in these livers. However, if the assumption is made that the extinction coefficient and molecular weight of the polar bound dyes are of the same order of magnitude as those of their precursors, then calculations show that after 6 weeks on a low riboflavin diet containing 0.06 per cent of the dimethyl dye the amount of polar bound dye in an average rat liver (7 gm. fresh weight) was of the order of 40 μgm. On a similar basis the amount of polar bound dye in the liver of a rat fed 0.106 per cent of *p*-aminoazobenzene would be about 6 μgm.

Free and bound aminoazo dye in liver tumors.—In these experiments the livers of rats bearing tumors which had arisen and grown during the continuous feeding of *p*-dimethylaminoazobenzene were perfused and the tumorous and non-tumorous portions separated. Only small non-necrotic tumors were used and the areas containing doubtful tumors were discarded. The relatively normal tissue and the tumors were then analyzed for free and total bound aminoazo dye. The results given in Table IV demonstrate that tumors arising in livers during continuous feeding of the dye always contained free aminoazo dye; in some cases the level of free dye in the tumor was as great as that of the surrounding liver, but on the average only 84, 32, and 86 per cent as much of the dimethyl, monomethyl, and completely demethylated dyes, respectively, were present. However, if the analyses were made after a rat with a tumor had been fed a dye-free diet for several weeks and was then again fed *p*-dimethylaminoazobenzene for 10 days only low levels of *p*-aminoazobenzene and no methylated dyes could be detected in the tumor although the levels of the 3 dyes in the

surrounding liver were approximately the same as usually obtained after feeding of the dye. In contrast to the surrounding normal liver the tumors from rats fed *p*-dimethylaminoazobenzene continuously contained no detectable quantity of bound dye: the uncorrected E values for tumors were 0.042 to 0.066 (av. 0.052) per 100 mgm. while the liver powder from rats not fed the dye had an average E value of 0.050. The acid-ethanol solutions of the tumor extracts were light yellow in color.

TABLE IV: THE LEVELS OF FREE AND BOUND AMINOAZO DYES IN LIVER TUMORS AND ADJACENT LIVER TISSUE
(*p*-Dimethylaminoazobenzene fed continuously for 4 to 6 months)

	Free aminoazo dyes (μgm./gm. fresh weight)					
	Liver			Tumor		
Sample	DAB	MAB*	AB*	DAB	MAB	AB
1	0.20	0.09	1.6	0.10	0.06	1.5
2	0.13	0.31	1.1	0.05	0.18	1.9
3	0.24	0.24	1.9	0.23	0.13	0.3
Average and range of 8 samples including 1-3	0.19 (0.07-0.39)	0.22 (0.09-0.39)	1.5 (0.5-3.0)	0.16 (0.04-0.33)	0.07 (0.00-0.18)	1.3 (0.2-2.6)
	Bound dye E uncorr./100 mgm. powder					
4		0.176			0.066	
5		0.208			0.048	
6		0.184			0.050	
7		0.195			0.042	
Av. (4-7)		0.191			0.052†	

* DAB = *p*-dimethylaminoazobenzene; MAB = *p*-monomethylaminoazobenzene; AB = *p*-aminoazobenzene.
† Normal liver powder = 0.050.

DISCUSSION

This appears to be the first report of a firm combination *in vivo* of derivatives of a carcinogen with a cellular constituent of high molecular weight. Weigert and Mottram (29) observed that the skin of mice painted with 3,4-benzpyrene contained a metabolite of the carcinogen which could be extracted from the tissue only after alkaline hydrolysis. These workers did not suggest that this derivative might have been bound chemically to a larger molecule, such as protein, but it seems quite possible that they were dealing with such a complex. In view of these observations further searches for a combination of carcinogen with cellular constituents should be profitable.

The present data suggest that the cellular constituent combined with the aminoazo dyes is protein in nature. At least 97 per cent of the final glycogen—and lipid-low crude protein precipitate used for analysis consists of protein and nucleoprotein, and the digestion of the powder with alkali or trypsin liberates the dyes at a rate which probably parallels the rate of destruction of the protein. Furthermore, two different methods of protein precipitation, heat coagulation and acid precipitation, yielded preparations of liver proteins which could not be freed of the bound dyes by hot ethanol extraction. In view of the postulated importance of nucleoproteins to the carcinogenic process one might expect the dyes to be combined with a nucleoprotein. However, if this is the case, the dyes must be combined with the protein through linkages which would be unaffected by the removal of the nucleic acid, since when this was done no dye was liberated. As yet it is not proved that the bond between the aminoazo dyes and the liver protein is a covalent linkage although it seems unlikely that a salt-like union or an adsorption involving secondary linkages such as hydrogen bonds could resist the prolonged action of hot polar solvents, heat coagulation, a detergent, and hot trichloroacetic acid. The later reagent apparently disrupts both the salt and non-salt linkages which exist between nucleic acid and protein in nucleoproteins (1, 25). Furthermore, the highly polar character of the majority of the liberated dye indicates that some polar fragment released from the liver protein by alkali or trypsin may still be attached to the aminoazo dye. The question must await further information on the structure of the liberated polar dye.

The maximum level of bound dye in the rat liver was reached after 4 and 6 weeks on non-protective and protective diets respectively. This is in contrast to the free aminoazo dyes which reach a maximum after 1 week of feeding (10). When the dye was then omitted from the diet the levels of both the bound dyes and the free dyes dropped nearly to zero in 2 weeks. These facts suggest that considerably more bound dye is formed in the liver than is found at any one time. If this is true, the amount of bound dye found upon analysis would be the resultant of two reactions, with the rate of formation of the bound dye exceeding, perhaps only slightly, the rate of

destruction. Thus it appears that similar levels of bound dye could result from combinations of different rates of formation and destruction and that in comparisons between livers this possibility might have to be taken into account.

The virtual absence of the bound dyes from all of the rat tissues studied except the liver is an obvious correlation with the high specificity of the carcinogenic aminoazo dyes in producing only liver tumors. The ability of high dietary levels of riboflavin to lower the rate of accumulation and the level of bound dye in the liver agrees with the known protective effect of this vitamin (4, 17, 19). Since the level of riboflavin in the liver appears to be a factor common to many of the diverse dietary effects observed with *p*-dimethylaminoazobenzene (17), it is possible that a more basic factor common to these effects is the rate of accumulation of bound dye in the liver. Furthermore, the appearance of similar quantities of bound *p*-monomethylaminoazobenzene and *p*-aminoazobenzene and of apparently the same polar bound dye in the livers of rats fed either *p*-dimethylaminoazobenzene or *p*-monomethylaminoazobenzene correlates with the observed metabolic interchangeability and equal carcinogenic potencies of these two dyes (9, 16). Similarly, the non-carcinogenic dye, *p*-aminoazobenzene, a metabolite of the carcinogenic dimethyl and monomethyl compounds (16), gives rise to a very low level of a polar bound dye in the liver that appears to differ from that obtained with the methylated dyes. The absorption spectrum of the total bound dyes in this case suggests the presence of the original primary amino group. In this connection it is of interest that the methylation of *p*-aminoazobenzene *in vivo* has not been observed (9, 16). The high species specificity possessed by *p*-dimethylaminoazobenzene as a carcinogen also correlates with the absence of bound dye in the livers of rabbits, guinea pigs, and chickens. As yet tumors have not been produced in these species after prolonged feeding with *p*-dimethylaminoazobenzene (5, 21). A similar situation appears to exist in the case of the cotton rat although our period of observation for tumor development on this species extends only to 8 months. While mice develop liver tumors when fed *p*-dimethylaminoazobenzene, they do so more slowly than rats; the low activity of this dye in the mouse (5, 6, 24) agrees with the finding that mouse livers contain about one-third as much bound dye as is found in rat livers. Thus the apparent correlations observed between the level of bound dye in a liver and the likelihood of a tumor developing in that liver under the varied conditions studied suggest that the bound dyes have an important place in the carcinogenic process induced by *p*-dimethylaminoazobenzene. Hence either *p*-monomethylaminoazobenzene or an unidentified polar aminoazo dye, or both, may be the primary carcinogens when the parent carcinogen, *p*-dimethylaminoazobenzene, is fed and the formation of the dye-protein compounds may be one of the initial phases in this particular carcinogenic process. It is recognized of course, that the bound dyes isolated by the methods described above may not be the primary carcinogens themselves but derivatives thereof.

Such a theory of carcinogenesis by *p*-dimethylaminoazobenzene would have to explain why the amount of bound dye reaches a maximum after about 4 to 6 weeks and decreases thereafter in both protected and non-protected groups of animals. Since the end result of the process (the liver tumor) is a tissue containing no bound dye, it is possible to explain the decreasing amount of bound dye in the precancerous livers as a gradual removal of the proteins capable of combining with the dye so that eventually, in certain areas at least, there is no protein able to combine with the dye. While a similar decrease occurred in the protected livers, the rate of accumulation of bound dye and the levels attained were always below those observed in the non-protected group. Since by this hypothesis the absence of precancerous changes means that the proteins were not being removed by combination with the dye to the same extent in protected as in non-protected livers, it is necessary to assume an increased ability of the protected livers to destroy the dye. Indeed the data on the disappearance of the bound dye from the livers of rats fed diets high and low in riboflavin indicate that at least part of the protective effect of this vitamin against *p*-dimethylaminoazobenzene consists in hastening the destruction of the bound dye. Hence the "adaptation" observed in the rat liver towards the azo dye may be twofold and consist of a combination of (a) a decreased content of protein possessing the ability to combine with the dye, and (b) an increased ability to destroy the dye. It is possible that in livers low in riboflavin, process (a) is predominant and that in livers high in riboflavin, process (b) becomes a major factor. In the case of rats fed a diet low in riboflavin the occurrence of a maximum level of bound dye in the liver after 4 weeks followed by a slow decline may be related to the initial chromatolysis (loss of basophilic material, presumably nucleic acid) and the resultant chromatogenesis (regeneration of nucleic acid) observed by Opie (20) in the livers of rats fed *p*-dimethylaminoazobenzene under similar conditions and times. Although similar changes in

the bound dye content were observed in the livers of the protected rats, such cellular changes probably occurred later and at a much lower intensity in these livers; in fact this was the case observed by Opie in a group of rats fed a partially protective diet.

Since both normal liver and liver tumors contain the free aminoazo dyes when *p*-dimethylaminoazobenzene is fed, the absence of bound dye observed in the tumor that arises in a liver containing considerable amounts of bound dye is intriguing. This finding is in harmony with the fact that the carcinogen is no longer needed once a tumor has been initiated and suggests that the tumor cell and its probable precursor, the normal liver cell, differ with respect to certain proteins that are concerned in the induction and possibly the maintenance of the malignant state. The idea that the significant change in carcinogenesis is an alteration in protein has been suggested by others; *e. g.*, by Lavik, Moore, Rusch, and Baumann (7), on the basis of quite different evidence. In order that the change in protein be heritable it seems necessary to assume that the proteins so affected are autosynthetic (23) and hence would involve directly certain genes, plasmagenes, or possibly certain enzymes. The recent papers by Spiegelman (26, 27) are of particular interest in this regard. The combination of the carcinogen and an autosynthetic protein could result in the gradual removal of this protein from the cell with or without the rise of other proteins in the cell. Thus the deletion of a protein may upset a competitive balance of proteins that could lead to the dominance of the cell by a self-duplicating protein concerned only with continual growth (23). Or the formation of a carcinogen-protein complex may lead not only to the loss of the protein but this foreign complex may attempt to duplicate itself with the result that a new self-reproducing protein is formed. Although such entities would be termed viruses if proven isolable and carcinogenic it is obvious that these proteins could maintain the malignant state and still not satisfy these experimental criteria. In any event *p*-dimethylaminoazobenzene might initiate the carcinogenic process through sublethal combinations of this dye or its metabolites with critical proteins in normal liver cells and their descendants. The autonomous tumor may be the eventual outcome of some of the damaged liver cells through a permanent alteration or loss of proteins essential for the control of growth but not for life.

SUMMARY

1. The livers of rats fed the hepatic carcinogen *p*-dimethylaminoazobenzene have been found to contain aminoazo dyes bound tightly to a cellular constituent, probably protein.

2. A preparation of crude liver powder is described; at least 97 per cent of this powder is protein plus nucleoprotein. This powder contains the bound dyes and no detectable quantity of free aminoazo dye. The bound dyes were not removed from such preparations by boiling organic solvents or by hot trichloroacetic acid which removes all of the nucleic acid. The dyes have been released only by prolonged tryptic or alkaline hydrolysis.

3. The bound dyes liberated by alkaline hydrolysis were fractionated by solvent extraction into non-polar and polar dyes. The non-polar dyes were identified as *p*-monomethylaminoazobenzene and *p*-aminoazobenzene. An unidentified polar dye accounted for approximately 90 per cent of the color of acid solutions of the total bound dyes.

4. Quantitative methods are described for the determination of the bound dyes in liver powders. In the determination of total bound dye the dyes were released by hydrolysis in hot ethanolic alkali, extracted therefrom with ethanol-ethyl ether, and increased by their color in acid-ethanol at 520 mμ.

5. No bound dye was found in rat tissues in which *p*-dimethylaminoazobenzene does not induce tumors, *viz.*, small intestine, kidney, spleen, lung, heart, and skeletal muscle. While no bound dye was found in the red blood cells, low levels of bound dye were found in the proteins of the blood plasma.

6. When either *p*-dimethylaminoazobenzene or its carcinogenic metabolite, *p*-monomethylaminoazobenzene, was fed to rats, the same bound dyes appeared to be present in the liver in similar amounts. Only bound *p*-aminoazobenzene and very low levels of an apparently non-methylated polar bound dye were noted in the livers of rats fed the non-carcinogenic *p*-aminoazobenzene.

7. Low levels of bound dye were found in the livers of mice; this species develops liver tumors slowly. No bound dye was found in the livers of guinea pigs, rabbits, cotton rats, or chickens; these species are resistant to the carcinogenic action of *p*-dimethylaminoazobenzene.

8. When the carcinogen was fed to rats, the level of bound dye in the liver reached a maximum after about 4 to 6 weeks of feeding and thereafter slowly decreased. Throughout a dye-feeding period of 5 months the level was lower in the livers of animals fed a protective diet high in riboflavin than in those fed a non-protective diet low in riboflavin. When the dye was omitted from the diet, the level of bound dye fell nearly to zero in 2 weeks; the rate of disappearance was greater when a diet containing a high level of riboflavin was

substituted for a low riboflavin diet after the feeding of *p*-dimethylaminoazobenzene was stopped.

9. The bound dyes were not found in tumors arising in livers which contained considerable levels of these dyes.

10. The correlations observed between the level of bound dye in a liver and the likelihood of a tumor developing in that liver suggest that the bound dyes have an important place in the carcinogenic process induced by *p*-dimethylaminoazobenzene. Thus either *p*-monomethylaminoazobenzene or an unidentified polar aminoazo dye, or both, may be the *primary* carcinogens when the *parent* carcinogen, *p*-dimethylaminoazobenzene, is fed and the formation of the dye-protein compounds may be one of the first steps in the carcinogenic process. The autonomous tumor could be the eventual outcome of some of the damaged liver cells through a permanent alteration or loss of proteins essential for the control of growth but not for life.

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